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Characterizing Salmonella fecal shedding among racehorses in Louisiana

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CHARACTERIZING *SALMONELLA* FECAL SHEDDING AMONG RACEHORSES IN LOUISIANA

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bp - base pair

C - centigrade

CO_2 - Carbon dioxide

CFU- -colony forming units

dATP - dexoxyadenosine triphosphate

dCTP - deoxycytidine triphosphate

dGTP - deoxyguanosine triphosphate

dNTP - deoxynucleotide triphosphate

dTTP - deoxythymidine triphosphate

LVMDL - Louisiana Veterinary Medical Diagnostic Laboratory

$MgCl_2$ - magnesium chloride

ml - milliliters

mM - millimolar

nM - nanometer

PCR - polymerase chain reaction

rpm - revolutions per minute

UV - ultraviolet

µl - microliters

µM - micromolar

ABSTRACT

Salmonella is an important intestinal pathogen in horses capable of infecting populations without demonstrating clinical illness. This study was performed to determine the prevalence of *Salmonella* fecal shedding among racehorses in Louisiana. Three serial fecal samples were collected from 429 Thoroughbred horses housed at four racetracks. Feces were tested for *Salmonella* by microbiologic culture with selective primary enrichment and delayed secondary enrichment (DSE). Samples were also evaluated for the presence of *Salmonella* by polymerase chain reaction (PCR) using genus-specific oligonucleotide primers. A total of 7 (1.6%) horses were positive for *Salmonella* by either primary bacterial culture or DSE and an additional 2 horses (0.5%) were positive for *Salmonella* by PCR. The combined prevalence of *Salmonella* fecal shedding from among all the horses in this study was 2.1%. The results of this study suggest that the prevalence of fecal shedding of *Salmonella* among racehorses in Louisiana is low.

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

Equine salmonellosis has long been recognized as an important infectious cause of colitis in adult horses.[1-3] The disease also has an important impact on other domestic species and man. Each year the Centers for Disease Control and Prevention (CDC) confirms approximately 40,000 human cases of salmonellosis [4], however there are an estimated 800,000 to 4 million cases of unconfirmed *Salmonella* infections each year with 500 of those cases being fatal.[5] The impact of *Salmonella* is felt heaviest in the food industry, which spends countless dollars on preventing the contamination of food-grade products and investigating outbreaks of disease.[6]

In the equine industry, the economic impact of salmonellosis can be substantial as well. Financial losses can be incurred by the horse owner through the cost of therapy for clinically affected animals and through the death of valuable horses. In some situations, a single case can lead to a large-scale disease outbreak, particularly in areas of large congregations of horses such as breeding farms, racetracks, or veterinary hospitals. In these circumstances, the financial and emotional toll may be catastrophic. While the exact costs of nosocomial *Salmonella* disease outbreaks nationwide are not known, published estimates for individual centers have ranged from $10,000 to $2 million.[7-9] While these numbers are well below the estimated cost of nosocomial infections from human hospitals (National Foundation for Infectious Disease), they represent a substantial loss of revenue and are damaging to the institutional reputation. As a result of this threat, most veterinary hospitals take a pro-active approach to prevent the introduction of disease by instituting biosecurity measures, rather than waiting for an epidemiologic disaster to occur.

Beside the financial threat, equine salmonellosis also poses a zoonotic risk to humans who handle the affected animals. One study found *Salmonella* was cultured from 27.7% of the

households of personnel with potential exposure in the workplace.[10] This implies that individuals with occupational exposure to *Salmonella* (laboratory workers, livestock workers, veterinary personnel handling infected animals) can transport the organism and contaminate the household environment. Despite this finding, documented zoonotic cases of salmonellosis in man are sporatic and outbreaks connected to animals are uncommon. Although documented zoonotic infections from horses are seldom reported, the CDC has estimated that approximately 6% of salmonellosis cases reported in the U.S. are associated with exposure to pet reptiles, most notably among children <5 years old, the elderly and the immunocompromised.[11] While solitary cases of *Salmonella* gastroenteritis are important, the most significant impact of the organism is felt when large-scale disease outbreaks occur. In human outbreaks, food and water-borne sources are usually implicated. A large nationwide outbreak of salmonellosis occurred in 1994 when a nationally distributed ice cream became contaminated with nonpasteurized egg-product from a common tanker trailer.[6] One recently documented outbreak of *Salmonella* infection in people was directly linked to a small animal veterinary hospital. Pulsed-gel electrophoresis confirmed that seven human *Salmonella* cases shared a common pattern to a *Salmonella* positive cat that was admitted to the hospital.[12] Therefore, examining *Salmonella* shedding among various populations of domesticated animal species, including horses, would provide useful information to protect the public.

***Salmonella* History**

Salmonella was named for a notable American veterinary pathologist, Daniel E. Salmon. Salmon was a member of the first graduating class of Cornell University in 1872, with a bachelor degree of veterinary science.[13] After working as a veterinarian, he was awarded the first doctorate in veterinary medicine in 1876. He distinguished himself as a leader in animal diseases by participating in a campaign to eradicate pleuropneumonia in cattle. For his efforts, he was

asked to help establish of the U.S. Bureau of Animal Industry and became its director in 1884. The study of modern microbiology was making historical discoveries in this post-Civil war era. During his tenure as director of the bureau, Salmon was awarded a grant of $10,000 to study hog cholera. As a successful leader, Salmon knew the importance of selecting equally qualified assistants. He chose Theobald Smith, a medical school graduate who was a tenacious and meticulous researcher. Smith's knowledge of microbiology techniques were self-taught, by studying the methods of his European peers Koch, Ross and Ehrlich. Working in the attic of the Bureau, Smith faced challenges dealing with melting media during the summer months. However, he went on to isolate "hog cholera bacillus" organism from pig intestines and co-authored the findings in a paper with his collaborator Salmon. As senior author, Salmon received the recognition and naming opportunity of the organism (*Salmonella)*, souring the relationship between the two scientists.[13]

Salmonella Morphology and Characteristics

Salmonella belongs to the family of bacteria called Enterobacteriaceae, which is comprised of facultative anaerobic, Gram-negative, bacillus (or rods). Structurally, most *Salmonella* possess long flagella which direct their movement, acting as a propeller for swimming. They also are covered with surface pilli, which are short, hair-like structures that are involved in cellular attachment. Like other Gram-negative bacteria, the outer membrane of the cell wall is composed of various structurally and functionally important molecules. One of these molecules is lipopolysaccharide (LPS), which is an important virulence factor for Gram-negative bacteria (discussed later). One portion of LPS, the O-specific polysaccharide tail, contains sugar variations which are used to identify different *Salmonella* types. These O or somatic antigens are heat stable and are exposed on the surface of the bacteria to the surface environment. Some capsulated *Salmonella* (*S.* Typhi and *S.* Paratyphi) also possess another surface polysaccharide,

the Vi antigen, which is heat-labile and may provide the organism protection from phagocytosis.[14]

The taxonomic nomenclature of *Salmonella* has suffered numerous revisions throughout the years. This has led to much confusion in scientific reports and publications. The current nomenclature is based on work performed by Crosa et al., who demonstrated with DNA-DNA hybridization that all salmonellae belong to a single species, *Salmonella enterica*.[15] The only exception is *S. bongori*, which is a non-pathogenic organism with distinct characteristics. Subsequently, *S. enterica* was categorized into six subspecies: *S. enterica* subspecies *enterica, S. enterica* subspecies *salamae, S. enterica* subspecies *arizonae, S. enterica* subspecies *diarizonae, S. enterica* subspecies *houterae,* and *S. enterica* subspecies *indica,*. These six subspecies may also be identified by Roman numerals I, II, IIIa, IIIb, IV and VI respectively. [4] *S. bongori* was originally designated subspecies V, prior its categorization as a separate species. For simplicity, however, it may be referred to as "subspecies V" in antigenic formula. Since 1968, salmonellae have been further subclassified into serovars based on the surface antigens, including the O antigens (somatic antigens), H antigens (flagellar antigens) and Vi antigens (capsular antigens). Currently there are over 2,500 characterized serovars of *Salmonella*.

In 2003, the Centers for Disease Control and Prevention (CDC) adopted the Kauffmann-White Scheme for identifying serotypes.[4] Under this system, salmonellae in subspecies I (*S. enterica* subspecies *enterica)* are named, while organisms in subspecies II through VI are identified by an antigenic formula: subspecies [space] O antigens [colon] Phase 1 H antigen [colon] Phase 2 H antigen. For example, *Salmonella* Chameleon now is designated *S.* IV 16:z4,z32:-. Serotypes missing a particular antigen are designated with a minus sign ("-"), as in the above example. Despite these improvements, a debate continues in the medical community over the current nomenclature of *S.* Typhi, the causative agent of typhoid fever. Some feel that

the name *Salmonella enterica* subspecies *enterica* serotype Typhi, may cause physicians and researchers to confuse this human-adapted serotype that causes systemic infection with those that cause syndromes of gastroenteritis or colitis.

Serotyping has become an important tool for the National *Salmonella* Surveillance System to tract *Salmonella* isolates for epidemiological surveillance and to investigate disease outbreaks. According to data compiled by the CDC, the four most commonly isolated serotypes from clinically ill horses in 2004 were *S.* Typhimurium, *S.* Newport, *S.* Reading, and *S.* Agona.[4] *Salmonella* Newport was also the most common serotype isolated overall from clinical samples from domestic species and wildlife. In Louisiana, the most common serotypes isolated from people in 2004 included S. Newport, S. Mississippi, and S. Javiana. Interestingly, S. Mississippi was the serotype with the greatest increase in prevalence in previous 10 years (1994-2004).[4] The most commonly isolated serovars from non-clinical samples varied and included *S*. Newport (horses), *S*. Cerro (cattle), *S*. Heidelberg (chickens), *S*. Hadar (turkeys), and *S*. Derby (pigs).

Some *Salmonella* serotypes have a narrow host range or are adapted to a specific host, while others have a broad range of potential hosts. Some of the host-adapted serotypes include *S*. Typhi and Paratyphi A (humans), *S*. Gallinarum (poultry), *S*. Cholerasuis (pigs), *S.* Abortusovis (sheep) and *S.* Dublin (cattle).[16] Currently there are no host-adapted serovars in horses, dogs or cats. The molecular mechanisms that are responsible for a host restriction of some serotypes are poorly understood, but most believe there is a repertoire of adhesin molecules expressed by *Salmonella* that determine host surface recognition.[17] Salmonellae may have evolved host specificity through a series of gene transfers and deletions. Studies in pigeons of the host adapted serovars *S.* Typhimurium DT2 and DT99 demonstrate point mutation or small deletions in the genome.[18] Others have demonstrated that host-adapted serotypes like *S.* Typhi possess genomic insertions that may promote host restriction.[19] Certainly, the process that has

driven host adaptation appears complex, and therefore future studies should be aimed at genetic sequencing of many *Salmonella* serotypes for comparative analysis between host-adapted and ubiquitous strains.

Salmonella Pathogenesis

The primary mode of transmission of *Salmonella* is the fecal-oral route, however, airborne transmission is also possible. Oliveira et al found that pigs could be experimentally infected by inhaling *Salmonella* over short distances.[20] In both poultry and pig production facilities, methods to reduce the formation of aerosolized dust have reduced the transmission of *Salmonella* among resident populations.[20, 21] Both non-specific and specific host defenses attempt to quell infection with pathogenic organisms. After ingestion, the organism must survive the acid mileau of the gastric fluid. The vast majority of the bacteria perish in the stomach, but with a large inoculum, enough bacteria survive to reach the distal small intestine and colon. One study in mice, suggests that gut luminal contents and composition may be critical to establishing infection in the intestinal epithelium.[22] *Salmonella* Typhimurium failed to infect mice intestinal cells in vitro when inoculated in phosphate buffered saline (PBS), but invasion was enhanced with Luria-Bertani (LB) broth. The authors speculate that amino acid supply may be an essential signal for cellular invasion, since addition of tryptone and yeast extract to PBS (two ingredients of LB broth), resulted in epithelial infection by *Salmonella*. This suggests that salmonellae receive environmental cues that change their extra cellular lifestyle and invade target intestinal epithelial cells.

Although the exact mechanism of *Salmonella* invasion into intestinal epithelium is unclear, studies have documented early cellular invasion in swine using electron microscopy. [23] Bacteria preferentially adhered to microfold (M) cells associated with the follicle-associated epithelium (FAE) initially, but later were observed invading other cell types as well (goblet cells

and enterocytes). Their study suggests that M cells are targeted early in disease since they lack surface barriers such as glycocalyx and mucus, which is present on absorptive enterocytes and goblet cells.[24] Bacteria were also observed migrating through crevices formed by extruded enterocytes (a process of normal cell turn over), suggesting *Salmonella* takes advantage of these sites for invasion as well (Figure 1).

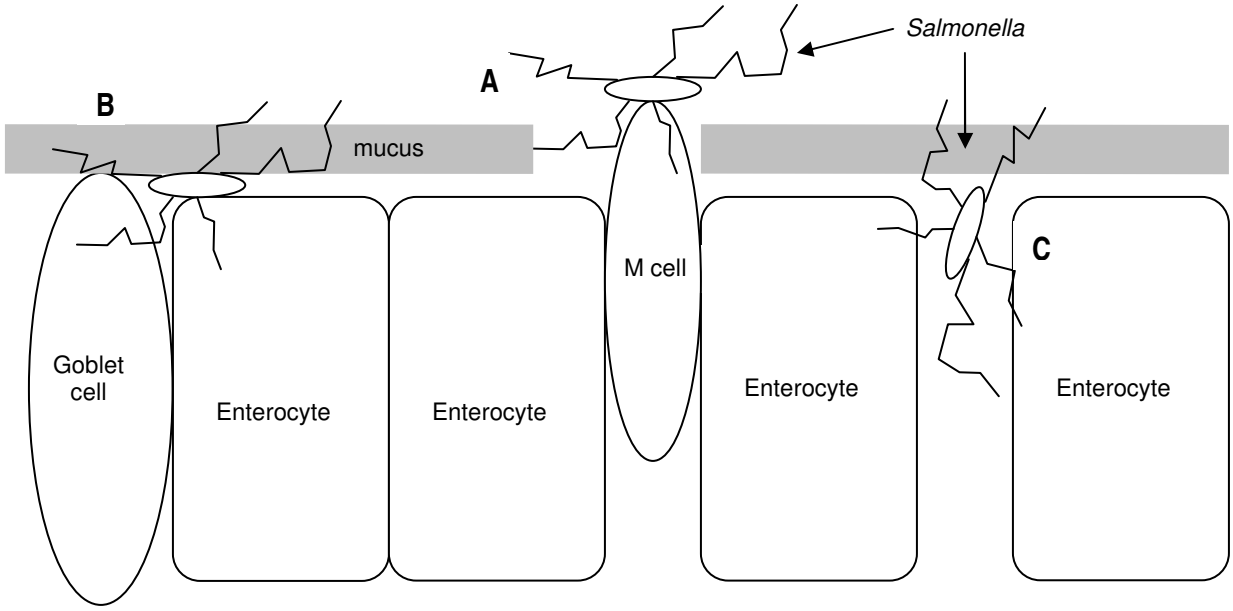

Figure 1: Various mechanisms for *Salmonella* invasion. Early in cellular invasion, *Salmonella* adheres and preferentially targets epithelial M cells (A) which lack surface barrier of mucus and glycocalyx. In later stages of infection, *Salmonella* can be observed invading enterocytes and goblet cells (B) or migrating through crevices formed by extruded enterocytes.

Bacteria have evolved unique mechanisms for invading non-phagocytic host cells. This "forced entry" into cells allows pathogens to evade host defenses and invade target tissues. There are a multitude of gene products that are required for full virulence and host interaction. In enteric bacteria, these genes are arranged into large clusters on the chromosome called pathogenicity islands (SPI) so the proteins they encode can be efficiently produced at the correct time and location. *Salmonella* spp. possess five pathogenicity islands, each encoding different

virulence factors that are needed for different phases of bacterial invasion. *Salmonella* pathogenicity island 1 (SPI1) is necessary for bacterial invasion of the intestinal epithelial cells (intestinal phase), SPI 2, 3, and 4 are required for bacterial growth and survival inside the host (systemic phase), and SPI 5 mediates intestinal inflammation and fluid secretion. Finally, some virulence genes are located extrachromosomally on a highly conserved plasmid and appear to promote bacterial growth and prolonged survival in the host. *Salmonella* pathogenicity island-1 is the most studied of the five SPIs and is responsible for encoding the type III secretion system (TTS). The TTS is a series of proteins that assemble to form a "molecular syringe" to gain access to eukaryotic cells and introduce effector proteins (Figure 2).

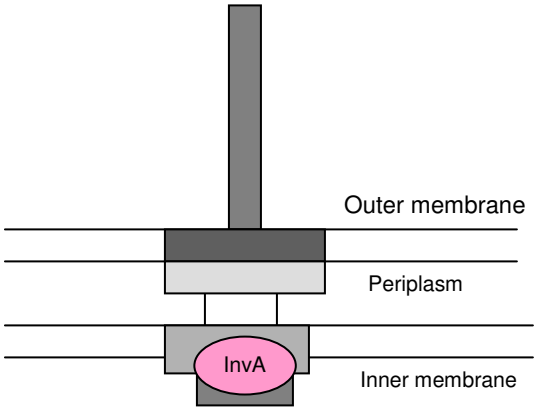

Figure 2: Depiction of Type III Secretory System (TTS) utilized by *Salmonella* to invade eukaryotic host epithelium. The protein encoded by the invasion A gene *invA* is shown.

The first step to *Salmonella* invasion is the attachment of adhesion pili to the surface of intestinal mucosal cells. Differences among adhesion pili have been identified among various serotypes.[25] Physiologic or environmental cues cause bacteria to sequentially upregulate the components of SPI 1, including the subunits of the TTS system and complex hierarchy of effector proteins.[26] *In vitro*, the optimal conditions for the expression of the TTS include slight

alkalinity, low oxygen and high osmolarity.[27] Once formed, the type III secretion system allows bacteria to create a hollow "pipeline" spanning both the bacterial and host cell membranes. The channel created allows the bacteria to deliver effector proteins directly into the host cell cytosol and enter the cell through special folding or attachment to chaperone proteins. These proteins often resemble the host's own proteins, thus allowing them to evade the cellular defense systems.[28] Some of the translocated proteins initiate signal transduction pathways and promote actin cytoskeletal rearrangements, creating a characteristic "membrane ruffling". Further plasma membrane manipulations create invaginations and eventually seal off around the invading microorganism. This is often termed macropinocytosis since it resembles the normal cellular mechanism of pinocytosis, but on a much larger scale.

Once internalized, *Salmonella* have evolved mechanisms for avoiding degradation by lysosomal vesicles. The *Salmonella* containing vesicle (SCV) continues to manipulate the host cell by recruiting actin molecules to the vacuole surface. This actin coat may act as a protective barrier preventing fusion with host oxidase-containing vacuoles. Secluded in the SCV, the pathogen can replicate by harnessing components of the host cell. Translocation of bacteria to adjacent cells can occur across the basolateral border trafficking them into deeper structures of the lamina propria. In this region, the bacteria gain access to phagocytic cells, especially tissue macrophages for invasion and eventual dissemination to other organs. One study demonstrated that the spread of *Salmonella* from the intestinal mucosa to the liver and spleen is dependent on CD18-expressing macrophages and possibly dendritic cells.[29] Infected phagocytic cells may allow cell mediated immune mechanisms to contain the infection. In neonates or the immunocompromised host, extraintestinal infections can occur resulting in septicemia, pneumonia, septic arthritis or meningitis. Some speculate that infected phagocytes may play a permissive role in allowing state of latent persistent infection.[30, 31] Studies in mice have found

Salmonella Typhimurium bacteremia peaked 30 minutes after gastrointestinal inoculation [29], and *Salmonella* can be identified in the ileocecal lymph nodes in esophagotomized pigs within 6 hours of intranasal inoculation [32] or with 24 hours after intragastric inoculation in mice.[33] An experimental infection of *Salmonella* Typhimurium in ponies revealed positive cultures of the mesenteric lymph nodes 20 hours after surgical inoculation of the dorsal colon. [34]

Mechanisms of Diarrhea

The classic feature of clinical salmonellosis in most vertebrate species is profuse, voluminous diarrhea. This occurs as a result of intestinal fluid losses by two mechanisms: (1) active fluid loss through secretory hyperstimulation and (2) passive fluid loss by inflammation-mediated malabsorption (Figure 3). *Salmonella* produces various virulence factors including exotoxin, cytotoxin, enterotoxin and endotoxin that mediate the development of diarrhea.[35] Cytotoxin, as the name implies, causes intestinal epithelial cell damage either directly through chelation of cations in the mucosal cell membrane or indirectly via stimulation of cytokines and inflammation. By inflicting direct damage of absorptive villous enterocytes, bacterial cytotoxins lead to reduced absorptive capacity and result in loss of electrolytes and water. Another mediator of inflammation is bacterial LPS or endotoxin, which through its interaction with local macrophages triggers a profound inflammatory effect resulting in the influx of neutrophils. Inflammatory mediators released by infiltrating leukocytes, such as cytokines, enzymes and oxygen species, provoke local tissue damage and contribute to the breach in mucosal integrity and intestinal malabsorption.

Bacterial enterotoxins initiate diarrhea by binding to receptors that stimulate the second messenger systems of cAMP and cGMP, which secondarily activate enterocyte fluid hypersecretion.[36] Cholera toxin (produced by *Vibrio cholerae)* is the prototypic secretagogue entertoxin. It activates the enzyme adenylate cyclase, leading to an increase of intracellular

cAMP and activation of luminal chloride pumps. The active transport of chloride into the intestinal lumen is followed by the loss of sodium, potassium and water, creating alterations in osmotic and electrical gradients. Experimentally, *Salmonella* enterotoxin is not secreted by the bacteria and must be extracted from viable cells, therefore it is believed that the toxin is liberated after enterocyte invasion to promote its physiologic effects.[37] Experimental inoculation of rabbit small intestine with viable *Salmonella* or bacterial lysaste resulted in enterotoxin activity as evidenced by increased intraluminal fluid accumulation.[38] However a similar *in vitro* study of equine colonic mucosa with a similar *Salmonella* inoculation did not result in significant fluid accumulation nor increases in mucosal cAMP.[34] This suggests that the equine colon may respond differently to *Salmonella* enterotoxin than the small intestine of other species. Microscopically, this study found a pronounced inflammatory response in equine colon segments inoculated with viable *Salmonella.* It is possible that such an inflammatory response may also lead to hypersecretion by triggering prostaglandin-dependent pathways via PGI_2 and PGE_2 (Figure 3) and activation of the enteric nervous system (both via the second messenger system).[36] Grondahl et al demonstrated that *Salmonella* Typhimurium was able to induce hyperosmotic fluid accumulation and increase intraluminal 5-hydroxytyptamine (5-HT) and PGE_2 in porcine small intestinal segments in response to *Salmonella* inoculation.[37] This response was blocked by the addition of the $5\text{-}HT_3$ receptor agonist, ondansetron. Unforutunately, many studies examine the physiologic response to *Salmonella* in jejunum and ileum (enteritis), but fewer studies examine colonic pathology (colitis), which may be why variable responses are seen between models in pigs, mice, rabbits and horses. One study of colonocytes in mice demonstrated that pathogens like *Salmonella* cause transient up-regulation of colonocyte receptor galanin-1 expression. When this receptor is activated by its ligand, galanin, which is found in enteric nerve terminals of the gastrointestinal tract, excessive colonic

secretion is observed.[39] So it is evident there may be other pathways in the pathology of salmonellosis which may be elucidated in the future.

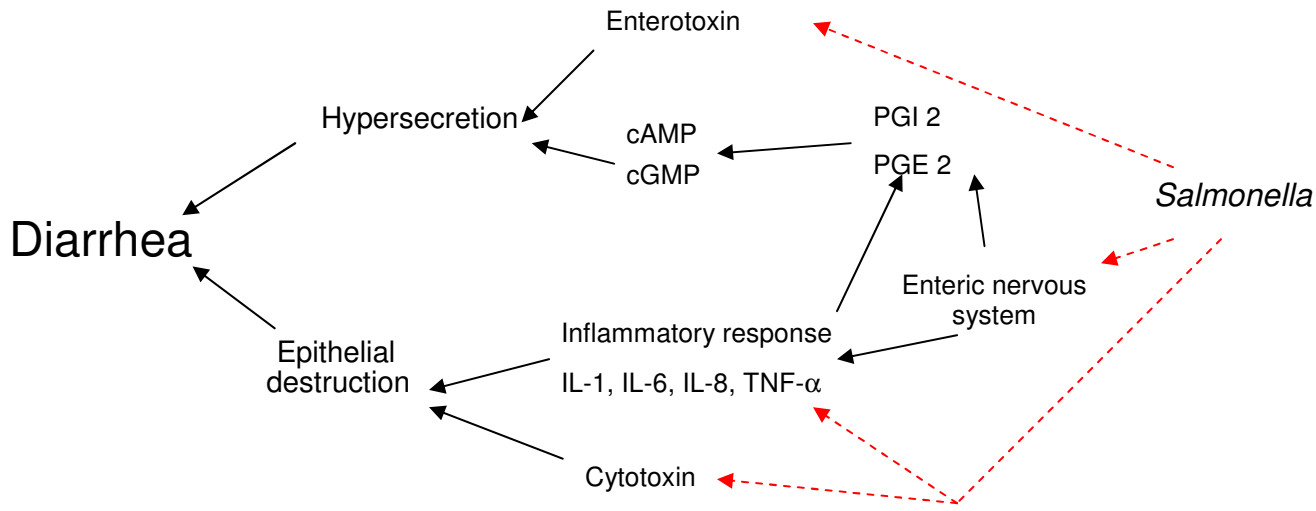

Figure 3**:** Diagram of the various mechanisms that contribute to the development of diarrhea by *Salmonella.*

Four clinical syndromes of equine salmonellosis have been described: 1) asymptomatic infection, 2) mild infection (fever, anorexia, and depression), 3) severe acute diarrhea, and 4) bacteremia.[40] Clinical salmonellosis is characterized by explosive and voluminous diarrhea, abdominal discomfort and systemic signs of toxemia including fever, mucous membrane injection with prolonged capillary refill time (CRT), anorexia, and depression. Accompanying signs of circulatory shock may be present especially if infection has advanced to bacteremia, including tachycardia, cool extremities, poor pulse quality and weakness. Milder infections are usually self-limiting, and patients may improve clinically in a relatively brief period of time. An important clinical feature of salmonellosis is the potential for some infected animals to shed the organism without demonstrating clinical signs of the disease (silent shedder). These inapparent carriers are capable of introducing the organism (albeit often in low numbers) into the environment of other susceptible horses. [41]

Therapy for acute salmonellosis is largely supportive. Aggressive intravenous fluids, treatments aimed at ameliorating the effects of endotoxemia (nonsteroidal anti-inflammatories, plasma, antisera, polymyxin B), and intestinal protectants are the mainstay of therapy. Probiotic therapy with commercial *Lactobacillus* preparations has shown anecdotally benefits in salmonellosis through the production of inhibitory factors and competition for mucosal colonization. However, experimentally, administration of probiotics to postoperative horses with colic did not protect against clinical salmonellosis nor prevent *Salmonella* shedding in several studies.[42] Probiotic organisms of equine origin have shown an inhibitory effect on the growth of *Salmonella in vitro* [43], and may prove to be a more promising therapeutic. Antibiotic therapy in patients with *Salmonella* colitis remains a controversial area. Antibiotics are not indicated unless the patients are at high risk of developing septicemia, such as in the case of neonates or other immunocompromised patients. Many suspect that antibiotics may in fact prolong the period of bacterial shedding and there is increasing evidence that inappropriate antibiotic use may contribute to the emergence of drug resistant salmonellae through bacterial selection pressures (discussed later).

Most horses that recover from acute salmonellosis or subclinical infection will shed the organism transiently for several days to weeks. One study followed the long-term shedding of horses infected with *Salmonella* and found 91% of the horses shed the organism for less than 120 days.[44] To confirm the end of the shedding period, five consecutive negative fecal cultures are recommended.[44] True persistent latent carriers are probably rare in horses, unlike other species like cattle, poultry and pigs. While a horse is shedding *Salmonella,* cautionary measures are recommended to prevent the development of salmonellosis in other susceptible animals on the farm. These include isolating infected horses until five negative cultures are obtained, wearing protective clothing/gloves, frequent handwashing, and complete cleaning and disinfection of all

equipment (tools, buckets, grooming implements, etc) and stalls. *Salmonella* organisms cannot be completely eliminated from either the hospital environment or the farm. However, limiting the exposure and spread of infection can help to decrease the incidence and severity of acute salmonellosis among horses.

In horses, outbreaks have occurred at veterinary referral hospitals, on breeding farms and at racetracks. Veterinary referral centers are of particular concern since congregations of potentially susceptible animals are subject to co-mingling in these facilities. Horizontal disease transmission is of particular concern in these situations. There have been numerous reports of *Salmonella* outbreaks among veterinary hospitals, and the majority of these infection outbreaks are due to multi-drug resistant strains of *Salmonella*, including the serotypes *S.* Saint-paul [45], *S.* Agona [46], *S.* Newport [9], *S.* give [47], *S.* Anatum [47-49], *S.* Infantis [50] *S.* Typhimurium [51, 52], *S.* Heidelberg [53], and *S.* Enteritidis [54].

Factors Influencing Infection

There are a variety of factors that will influence the development of clinical salmonellosis in horses. Some of these factors are related to the microorganism itself, such as the infective dose of bacteria and the virulence of the individual strain. Other factors that influence infectivity are related to the host and its individual susceptibility. Studies in mice have demonstrated that the number of *Salmonella* Enteritidis organisms required to infect conventionally raised animals is 10^6 or greater. [33] However, in germ-free mice, only 10 bacteria were necessary to result in diarrhea, septicemia and death. [33] Likewise, a study of experimental *Salmonella* infection in horses revealed that horses administered a dose of 1.5×10^7 organisms developed a febrile response without diarrhea. However, exposure to an infective dose of 1.5×10^{11} organisms resulted in clinical signs of acute colitis (fever, diarrhea, mental depression).[40] The equine alimentary tract contains 120 to 150 liters of ingesta and the typical horse excretes 14 liters of

feces per day. [55] With severe diarrhea, several liters of feces can be lost every hour. A horse with clinical salmonellosis typically sheds $10^4 – 10^5$ organisms per gram of feces [56], therefore with large volumes of diarrhea the level of environmental contamination can become significant.

Salmonella virulence is also dictated by a number of cellular components of the bacteria itself, some of which were mentioned earlier. These include adhesion molecules, toxins (cytotoxin, endotoxin, enterotoxin) and antimicrobial resistance. Some virulence factors are encoded in the microbial chromosome and some are located on extrachromasomal plasmids. Bacteria can acquire new virulence factors or modify their existing ones by two methods: spontaneous genetic mutations and transfer of DNA among bacteria. Spontaneous mutation are not common, however, because bacteria have a short generational interval, mutations can be propagated exponentially. Fortunately, spontaneous mutations do not impact virulence factors and antimicrobial resistance patterns to a large degree. [8] Unfortunately, bacteria can also share virulence factors by horizontal gene transfer with other bacteria. Bacterial conjugation is the transfer of genes by cell to cell contact usually by means of a pillus. Bacterial transformation is the ability of bacteria to acquire environmental DNA across the cell membrane and integrate it into the genome. Transduction is the final method for acquiring genes and it involves acquisition of genes by a vector such as a bacteriophage. Through these methods bacteria can effectively acquire resistance genes not only from fellow *Salmonella,* but different bacterial genera as well. In recent decades, the emergence of highly-virulent, multidrug-resistant *Salmonella* strains has become of great concern. There is speculation that the widespread antimicrobial use in veterinary hospitals may aid in the emergence and selection of multidrug resistant *Salmonella* which then lead to nosocomial disease. [8] The frequency and spectrum of *Salmonella* outbreaks may be increasing within veterinary referral centers. [57] Pulse-field gel electrophoresis (PFGE) determined that plasmid transfer from a nosocomial strain of *Salmonella* Krefeld was responsible

for the emergence of antimicrobial resistant *Salmonella* Saint-Paul in an outbreak of equine salmonellosis in California. [45, 58]

Studies of salmonellosis in horses at veterinary referral centers have identified risk factors that increase the likelihood of infection during hospitalization [7, 59-63] or during an outbreak [50, 51, 64]. Despite some inconsistencies between these studies, possibly due to the differences in sample populations and sampling techniques, many similar risk factors have been discovered. Horses receiving antibiotic therapy [59, 61, 63] feed restriction or change in diet [64] were at greater risk for developing salmonellosis possibly due to alterations in enteric microflora. Another study found that foals were at greater risk for infection [63] possibly due to reduced immunocompetency, lack of competing enteric microflora and coprophagia. Stress may also play a roll in *Salmonella* infection since several studies found prolonged transport [7, 65] or heat exposure [61] increased the likelihood of salmonellosis. Other stressors that have been identified include major surgery, particularly abdominal surgery [63, 66], and gastrointestinal disease (colic) [7, 59, 63] and respiratory disease [62]. Overall, these studies suggest that the factors that influence infection are not exclusive, and veterinary hospitals must maintain vigilant biosecurity measures to reduce nosocomial disease.

***Salmonella* Prevalence**

There are numerous studies examining the prevalence *Salmonella* shedding in a range of horse populations (Table 1). The reported prevalence among these studies is quiet varied. A study conducted by the United States Department of Agriculture, Animal Plant Health Inspection Service examined 8,417 horses from the U.S. general horse population for evidence of *Salmonella* by bacterial culture using a single fecal sample. The overall prevalence of active shedding at a given instance was in fact relatively small (0.8%).[67] Based on the study, it is believed that active shedding of *Salmonella* occurs rather infrequently in adult horses in the

general population. Others have suggested that latent carriers may account for the majority of horses infected with *Salmonella.*[30] One study found presence of the organism in 70% of the mesenteric lymph nodes of horses in slaughter establishments.[30] However, another study examined the mesenteric lymph nodes of horses that presented for necropsy at a veterinary teaching hospital and found very different results.[61] *Salmonella* was isolated from the lymph nodes in only 2% of the horses examined, suggesting that the slaughterhouse study may have over-represented the true prevalence of *Salmonella* infections among asymptomatic horses. It was hypothesized that the environmental contamination of the lymph node samples during sampling procedures or prolonged exposure of the slaughterhouse horses to environmental *Salmonella* may have resulted in the unusually high prevalence.[61] Therefore, it is plausible that the high prevalence of *Salmonella* in a single facility may not represent the prevalence of the organism in other equine populations.

Numerous studies have evaluated the prevalence of *Salmonella* fecal shedding among hospitalized patients in veterinary teaching hospitals in a variety of populations (Table 1). The reported prevalence has ranged from 1.7 to 10% among studies examining all horses admitted to veterinary hospitals. [7, 30, 57, 62, 64, 66, 68-73] Studies examining horses admitted to intensive care units (ICU) have described a range in prevalence of *Salmonella* shedding from 5.46% to 6.3% [60, 61] and those admitted for gastrointestinal disease (including colic) from 5.5 to 13% [63, 74, 75]. One study found the prevalence of *Salmonella* shedding in patients increased during hospitalization with 0.4% positive upon admission and 4.35% positive during hospitalization. [62]

Only one study has examined *Salmonella* shedding in racehorses. [76] In this study, fecal samples were examined for *Salmonella* by culture from 50 clinically normal horses and 14 horses with diarrhea. Positive cultures were obtained from 6 of the normal horses (12%) and none of the diarrheic horses. The combined prevalence was 9.4% when both sample groups

were combined, and the only serotype identified was *Salmonella* Tucson. Interestingly, all the *Salmonella*-positive horses were under the care of the same trainer, had an association with chickens (roaming in the stable area), and were under moderate to heavy training programs. However other horses that were culture negative for *Salmonella* also had an association with chickens, but were under the care of different trainers and were housed in a different area. This study concluded that the stress of racing and an association with poultry in the stable may increase the likelihood of salmonellosis in racehorses. [76]

Table 1: Studies of *Salmonella* shedding in horses.

Author	Year	Location	Detection Method	Population	Sample number	Prevalence
Smith	1978	California-Davis	Culture	General hospitalized	1451	3.2%
Palmer	1985	Pennsylvania	Culture	Admitted for Colic	100	13%
Begg	1988	Sydney, AUST	Culture	General hospitalized & Mares on farm	250 (hospital) 75 (farm)	2.8% (hospital) 0% (farm)
McCain	1990	Oklahoma	Culture (LN)	Slaughterhouse	70	71.4%
Traub-Dargatz	1990	Colorado	Culture	General hospitalized (>3 days)	246	7%
Cohen	1996	Texas	Culture and PCR	Outpatient	152	0% (culture) 26% (PCR)
Cohen	1996	Texas	Culture and PCR	Inpatient	110	10% (culture) 64% (PCR)
Bucknell	1997	Melbourne, AUST	Culture	Slaughterhouse	142	27%
Ravary	1998	Montreal, CANADA	Culture	General hospitalized	613	1.7%
Mainar-Jaime	1998	California - Davis	Culture	Hospitalized in isolation or ICU	1446	6.3 %
House	1999	California – Davis	Culture	Hospitalized in ICU	1429	5.46%
Kim	2001	Colorado	Culture	Hospitalized due to colic	246	9%
Ewart	2001	Michigan	Culture	Hospitalized with GI disease	638	5.5%
Alinovi	2003	Purdue	Culture and PCR	Isolation patients	34	26% (culture) 68% (PCR)
Alinovi	2003	Purdue	Culture	General hospitalized (in and outpatient)	232	0.5% (admission) 4.3% (during hospitalization)
Ward	2005	Purdue	Culture and PCR	Hospitalized for NON-GI disease	116	9.5% (culture) 75% (PCR)
Ernst	2004	Florida	Culture	Hospitalized with GI disease	1750	13%
Morley	2005	Colorado	Culture	General hospitalized	3695	2.4%

CHAPTER 2

ISOLATION AND IDENTIFICATION OF *SALMONELLA*

Bacterial Culture

The apparent gold standard for detecting *Salmonella* is bacterial culture. A study examining swine feces found that storage at 4°C for 6 days or -15°C for 14 days resulted in a lower portion of *Salmonella* isolation compared to same day processing. [77] However, when processing on the day of collection is not practical, this same study recommended storage at 4°C since freezing appeared to reduce viability by a greater magnitude. Laboratory techniques for isolating *Salmonella* are standardized in human medicine and in the food industry; however, the techniques utilized in veterinary diagnostics vary greatly. Selection of the appropriate media can be confusing since the recommendations vary depending on the specimen type (e.g. blood, feces, tissue, etc). Many clinical samples harbor a mixture of bacteria and therefore selective culture media is used to assist in the recovery of the target species. Strategies employed by selective media include inhibitor substances (such as metals, chemicals and antibiotics), which hinder the growth of undesirable bacteria, buffers that optimize the pH for certain microorganisms, supplements (vitamins, blood, serum etc) that enhance the growth of fastidious bacteria, selective carbohydrates (lactose, sucros, maltose, dextrose, xylose) as energy sources, proteins (peptone, casein, typtones) for metabolism and indicator substances.

Most protocols for isolation of *Enterobacteriacae* describe a primary enrichment step to enhance the growth of the certain bacterial species while inhibiting the growth of unwanted organisms. Some enrichment media for *Salmonella* include selenite broth, tetrathionate broth, Rappaport-Vassiliadis, and Gram-negative broth. Fecal specimens harbor a mixture of bacteria, however, coliforms and other intestinal flora predominate. Pre-enrichment of fecal samples is important to reduce the number enteric bacteria such as *Escherichia coli,* which can far outweigh

the number of other organisms, such as *Salmonella*, and improve the chance of isolating organisms that are few in number. Sodium selenite is inhibitory to *Escherichia coli* and other coliforms, and works by restricting these bacteria in lag phase, while allowing *Salmonella* to enter into log phase. Ideally, an enriched sample should be plated on a selective media within 24 to 48 hours since prolonged exposure to enrichment media will reduce the inhibitory effects and allow coliform overgrowth. The addition of 0.1% novobiocin in the enrichment media causes a reduction in the number of Gram-positive organisms, and will increase the ratio of *Salmonella/*non-*Salmonella* species.[78] Further differentiation of *Salmonella* is accomplished by inoculation on selective agar including *Salmonella-Shigella* (SS) agar, Hektoen enteric (HE) agar, brilliant green agar, SM-ID agar, xylose-lysine-deoxycholate (XLD) agar, xylose-lysine-tergitol (XLT-4) agar, Rambach agar and bismuth sulfite agar. Media such as *Salmonella-Shigella* agar, Hektoen agar and Rambach agar contain high bile salt concentration, which inhibits the growth of all Gram-positive bacteria and retards the growth of many coliforms. However, *Salmonella* grows well in the presence of bile salts (as is evidenced by gallbladder infection of humans). Other inhibitors that are included in selective media include brilliant green dye (in brilliant green agar and SMI-ID agar), sodium desoxycholate (in XLD agar), and the anionic surfactant tergitol 4 (in XLT-4 agar). Most agars have an indicator substance to assist in bacterial identification. *Salmonella*, among other species, are able to liberate hydrogen sulfide from sulfur-containing amino acids like cysteine or other sulfur-containing compounds. Once sulfide is liberated, it combines with hydrogen to form hydrogen sulfide. In the presence of heavy metals, such as iron, bismuth, or lead, hydrogen sulfide will form a black precipitate. Media such as *Salmonella-Shigella* agar, XLD, XLT-4, Hektoen, bismuth sulfite and brilliant green agar contain sodium thiosulfate as a sulfur source and ferric citrate as a heavy metal source. Very few media contain lead as a heavy metal source since it will prevent the growth of

many fastidious organisms. However, there are multiple species of *Enterobacteriacae* that produce hydrogen sulfide (*Salmonella, Citrobacter, Proteus),* and there are a few species of *Salmonella* (especially *Salmonella* Typhi) that fail to produce hydrogen sulfide. This makes hydrogen sulfide production an imperfect indicator of *Salmonella* growth. Therefore, newer media, such as Rambach agar, SM-ID agar and others, incorporate chromogenic enzyme substrates into their formulations for the detection of salmonellae. Bacterial enzymes, such as B-galactosidase in the case of *Salmonella,* convert the chromogenic substrate and produce a color change in the colony. With Rambach media, for example, salmonellae appear as a brilliant red, while other bacteria are blue, violet or colorless. Some media utilize multiple chromogenic mixtures for detection of multiple *Enterobacteriacae.* Unfortunately, chromogenic media are more expensive and may be cost prohibitive on large scale sampling procedures.

Further confirmation of *Salmonella* is accomplished by using a series biochemical tests. The more biochemical tests that are performed on a suspect colony, the more sensitive the results. Klinger iron agar (KIA) and triple sugar iron (TSI) agar establish the ability of the organism to ferment different carbohydrates. These agars contain glucose and lactose in a ratio of 1:10. Non-carbohydrate fermenters fail to convert either glucose or lactose to acid so the media, which contains phenol red as a pH indicator, remains unchanged. Bacteria which ferment glucose, but not lactose, will initially produce acid as a result of glucose fermentation. However when the glucose supply is exhausted, these organisms will utilize amino acids in the aerobic portion of the media (the slant), and revert the media to the alkaline color of red. Organisms that are capable of fermenting both glucose and lactose, will result in acid production (and a yellow color change) in both the superficial and deep portions of the agar. Sodium thiosulfate and ferrous sulfate are added for the detection of hydrogen sulfide production.

Additional testing examines the ability of bacteria to use lysine as a sole carbon source. One such media, lysine iron agar (LIA) is used to differentiate certain species of *Citrobacter* (lysine decarboxylate negative) from *Salmonella* (lysine decarboxylate positive). The media contain a small amount of glucose as an energy source and once this is exhausted, the decarboxylase enzyme is activated to utilize the amino acids present in the media. Decarboxylation of lysine, or removing a molecule of CO_2, produces a pH shift in the medium as the alkaline amines are formed. Bromcresol purple is the indicator substance which confirms alkalinization.

Further differential testing may be accomplished by examining for the presence of bacterial urease. In Christensen's urea agar, urea is hydrolyzed to produce ammonia. A color change from yellow to fuchsia red occurs as the pH changes. This test is useful for differentiating urease negative *Salmonella* from urease positive *Proteus* and some species of *Citrobacter.*

For increased accuracy, multitest strip identifications systems are available to confirm the identification of the *Enterobacteriacae*. Some of these systems require overnight incubation (API 20E, Biomerieaux, Durham, NC, USA), while others provide rapid results in as fast as 4 hours (MicroID, Remel, Lenexa, KS, USA).

Enrichment Techniques

The food industry has sought methods to improve the accuracy of microbiological methodology to reduce the incidence of food borne illness. Increased recovery of *Salmonella* from samples containing low levels the bacteria is crucial to the success of these quality assurance programs. Enrichment techniques have been shown to increase the isolation of *Salmonella* from various sources including poultry [79], swine[77, 80], cattle [81], and environmental sampling [79]. Various enrichment techniques have been described including prolonged

enrichment (extended sample incubation times), secondary enrichment (using two or more enrichments in series) and delayed enrichment (storage of enrichment samples at room temperature for a period of time). Delayed secondary enrichment involves a primary incubation in enrichment broth, with incubation at 37°C (or 42°C depending on the media) for 24 hours followed by further incubation at room temperature for 3-5 days. This is followed by subculturing into new enrichment broth and a second incubation at 37°C for 24 hours. One study in poultry demonstrated a stepwise increase in isolation of *Salmonella* from poultry samples and environmental swabs with increasing enrichment incubation times. [82] The optimum recovery of *Salmonella* was found with 5-day delayed secondary enrichment. One study in poultry found that the addition of novobiocin to the enrichment and plating media increased the recovery of *Salmonella* by reducing the growth of other bacteria particularly *Proteus* spp.[79] This same study found that only 58% of *Salmonella* isolates were detected after conventional 24 hour incubation in enrichment media, and 91% of isolates were identified with delayed secondary enrichment. Interestingly, 9% of the isolates were only identified with 24 hour incubation and not DSE, therefore the author concluded that DSE should be used in conjunction with 24 hour enrichment and plating. The mechanism responsible for the improvements in *Salmonella* recovery with delayed secondary enrichment is unknown; however, some speculate that the prolonged incubation at room temperature may favor the growth of the species in the lowest numbers. [80] Bacteria in large numbers reach stationary phase faster and then make up a larger proportion of dying cells, while the bacteria in lower numbers (e.g. *Salmonella)* have not yet reached stationary phase. Since bacteria in stationary phase have a longer lag phase when they are subcultured into fresh medium, the bacteria in smaller numbers have an advantage. Lower temperature (e.g. room temperature) may also slow the decline of bacteria in the stationary phase. [80]

Polymerase Chain Reaction

Polymerase chain reaction (PCR) has emerged as a rapid and sensitive diagnostic tool for the detection small quantities of target DNA. Certain conditions must be optimized for the ideal *Salmonella* PCR performance and accuracy. Two of the most critical factors are selection of the target sequence and primer design. The complete genomic sequence of *Salmonella* Typhimurium LT2 has been sequenced and compared to three related bacteria for gene distribution and homology.[83] Many *Enterobacteriacae* share common virulence genes and phylogentic similarities, therefore a target gene must be genus-specific for *Salmonellae*, and not shared by closely related bacteria. Primers are designed with certain characteristics. They should be at least 18-24 base pairs in length, contain 60% guanine and cytosine residues, have no internal secondary structure, not compliment each other (to prevent primer dimmer formation), and have melting temperatures that allow annealing at 55 to 65 °C. [84] Many oligonucleotide primer sets have been describe for the detection of *Salmonella*, including those associated with the invasion genes *invA* [85, 86] and *invE* [86], histidine transport operon *hisJ* [87], SPI1 invasion gene *hilA* [88], virulence plasmid gene *spv* [89], virulence gene *sipC* [90], enterotoxin gene *stn* [91], *ompC* [92], *spaQ* [93]. Recent studies describe a serotype-specific PCR method of differentiating *Salmonella* Pullorum and from other serotypes using the *rfbS* gene. [94] Most researchers agree that in the future more serotype-specific PCR assays will be developed for serotype identification.

The *Salmonella* invasion A gene (*invA*) has become one of the most popular PCR target sequences since it was first described by Galan et al. [95] *InvA* gene is located in *Salmonella* pathogenicity island-1 and its encoded proteins are components of the type III secretion apparatus (Figure 2) which appears to be important for epithelial invasion by many *Salmonella* species.[95] These same group of researchers discovered that mutant strains lacking *invA* gene were unable to invade cultured epithelial cells. Subsequently, Rahn et al (1992) described a

primer pair that amplified a 284-bp fragment the *invA* gene which was detected in 626 of 630 *Salmonella* strains examined. Also, this primer pair did not amplify DNA from 21 genera of non-*Salmonella* bacteria proving its lack of cross-reactivity. [85] Due to the small number of false negative reactions that were observed in Rahn's study, another group of investigators designed a primer set that encompassed the junction between the *invE* and *invA* genes that amplified a 457-bp fragment.[86] This PCR hybridization identified all *Salmonella* serotypes examined and improved the level of detection 10 fold compared to the previous assays (improvement of detection from 300 CFU down to 9 CFU). However, in this study, non-specific amplification was observed with 2 non-*Salmonella* organisms (*Yersinia pseudotuberculosis* and *Edwardsiella tarda).* Most recently, others have further improved on the *invA*-PCR method by targeting the 3' region of the *invA* gene which does not share nucleotide sequence similarities with other bacteria. [96] As a result, these modifications have improved the specificity of the *invaA*-PCR assay while maintaining a high level of sensitivity.

Diagnostic PCR poses special challenges since many biologic samples, such as blood, muscle or feces, contain substances that interfere with the PCR assay, either by inhibiting DNA polymerase or affecting the balance of nucleic acids. Fecal samples remain one of the most difficult biologic specimens for DNA extraction and amplification because they contain multiple components that can inhibit the PCR reaction, including bilirubin, bile salts, complex polysarccharides, metabolic products of hemoglobin, DNases and proteases.[97-99] Fecal composition of these inhibitors may vary based on species, states of disease (such as intestinal dysfunction) [100], diet or food constituents (glycogen, fats and calcium), and environmental contaminants (phenolic compounds and heavy metals).[97] Several studies have examined *Salmonella* by direct examination of feces in horses[92, 101], however these studies have not proved very sensitive. To avoid false negative results and increase the level of detection, some

PCR protocols include a 24 hour period of enrichment to dilute inhibitory substances. In a follow-up study by Cohen et al (1995), the sensitivity of the PCR method was improved 1000 fold (down to 10 CFU *Salmonella*/g of feces) with the addition of an overnight enrichment in tetrathionate broth [102]. Unfortunately, some enrichment media, such as selenite, contain bile salts that while selective for *Salmonella,* are inhibitory to DNA polymerase. Various techniques have been described for DNA extraction and most include three basic steps: cellular lysis (by boiling or use of detergents), extracting DNA from cellular and histone proteins (by phenol/chloroform, sodium acetate or ammonium acetate), and finally DNA precipitation and dissolution of salts (ethanol or isopropanol precipitation). Commercial kits are available that remove inhibitory substances and improve the purity of the DNA isolated. Most manufacturers will not supply complete information regarding the composition of these kits for proprietary reasons. The basic principles of the kits involve lysis of all the cellular material with detergents, removal of inhibitors with a polysaccharide mixture, digestion of all exogenous proteins, binding of the DNA to solid matrix, followed by washing and elution. One study that compared four commercial kits and a non-commercial guanidium/isothiocyanate/silica matrix method found that the QIAmp DNA stool mini kit[d] was the most effective method of DNA extraction for human fecal samples in terms of PCR performance. [103] Another study investigated the addition of amplification facilitators to reduce the effects of PCR inhibitors. [104] The researchers found that the addition of 0.6% bovine serum albumin to the reaction mixture reduced the level of PCR inhibition in feces, possibly by serving as a preferential target for fecal proteases thus sparing DNA *Taq* polymerase. [104]

Since its introduction, real time PCR technology has improved the accuracy nucleic acid amplification compared to the standard PCR assay. By using a dual labeled internal fluorogenic probe, which acts as a molecular beacon[105], in addition to the standard sequence specific

primers, this assay allows greater confidence in the identity of the amplified product. [106] It also has the added advantages of providing quantitative measurements and eliminates the need for labor-intensive post-PCR handling and detection. Real-time PCR assays to detect *Salmonella* have been described for clinical samples in many species including cattle, horse and dogs[93], humans[107], as well as environmental samples[108]. In one study, real-time PCR achieved an overall relative sensitivity of 100% and specificity of 98.2%. [93] The methods for detecting products by conventional PCR involve visualization of the appropriately sized DNA band on an agarose gel, whereas real-time PCR relies on fluorescence detection, which is more direct, rapid and highly sensitive. One study of real-time PCR, reported a sensitivity of 2 CFU of *Salmonella* per PCR reaction by targeting the 122-bp *himA* gene. [105] Finally, conventional PCR requires additional steps to confirm whether the amplified product is the predicted genomic sequence. This is accomplished by DNA probes (with Southern blotting), sequencing or restriction-enzyme digestion. Real-time PCR uses an internal fluorogenic probe that when bound to the target sequence is detected as a fluorescent signal during the reaction process. So the need for post-reaction handling is eliminated.

Comparison of Bacterial Culture and PCR

Bacterial culture will remain the "gold standard" for *Salmonella* detection, since it permits investigators to obtain antibiotic sensitivity patterns and serologic typing; however, PCR has emerged as a useful tool with some advantages. Bacterial culture methodology may involve 4 to 7 days to identify and confirm *Salmonella* [86], whereas some PCR methods allow rapid identification of the organism in 3 to 24 hours.[92, 102] This may provide useful in epidemiologic investigations, where identification of infected individuals is needed to make patient management decisions. PCR is also advantageous when the isolate maintains atypical culture

characteristics (lack of H2S production, lactose fermentation, etc) that may lead to misclassification by bacterial culture. [102]

Some studies with parallel comparisons of PCR and bacterial culture have reported that PCR has superior analytical sensitivity for detecting *Salmonella.* [57, 75] The reported sensitivities of PCR of equine feces has ranged from 10^3-10^4 CFU/g of feces [101], while culture has ranged from 10^2– 10^4 CFU/g of feces[101]. However, there are some major disadvantages associated with PCR. The assay itself can be technically challenging, since small mistakes in the concentration of the components ($MgCl_2$, primers, dNTPs, DNA polymerase, etc) may result in imbalances that alter of the reaction. Magnesium is a critical component of the assay since it complexes with dNTPs to allow their recognition by DNA polymerase. Excessive $MgCl_2$ may affect the fidelity of DNA polymerase and increased non-specific primer binding, whereas sequestration of $MgCl_2$ by chelators may inhibit amplification.[97] The amount of DNA polymerase is also critical since increased enzyme concentrations may lead to decreased assay specificity and fidelity.

Another major disadvantage of PCR is that non-viable *Salmonella* organisms may be detected, which can lead to misclassification of animals that are recovering from infection or may have acquired dead organisms by ingestion.[109] However, detection of non-viable organism may be useful when improper sample handling (such as drying, freezing, etc) renders the sample negative by bacterial culture.[102] Ewart et al (2001) demonstrated that disinfectants that disrupt the bacterial cytoplasmic membrane, such as ammonium chloride or phenol compounds, render the *Salmonella* DNA detectable by PCR testing on a variety of surfaces, but negative on bacterial culture.[75] Whereas disinfectants that degrade DNA, such as bleach or formalin, rendered most environmental surfaces negative by both PCR and culture. Another study discovered an overall higher proportion (49%) of environmental samples were positive by PCR compared to bacterial culture (2.1%), again suggesting that disinfectants may have resulted in bacterial death through

disruption of cellular membranes without degredation of chromosomal DNA.[71] Both studies concluded that *Salmonella* PCR should not be used for assaying environmental samples; however, it could be useful in assessing the residual level of environmental contamination after disinfection. [71, 75] A newer application, ethidium monoazide-PCR (EMA-PCR), may be useful in the future to distinguish viable and non-viable *Salmonella.* A study of *Campylobacter* in poultry found that ethidium monoazide can enter cells with damaged membranes and bind DNA covalently to inhibit the PCR reaction. [110] Thus with this methodology, only DNA from viable cells can be amplified by the PCR reaction.

CHAPTER 3

STUDY OF FECAL SHEDDING OF *SALMONELLA* AMONG RACEHORSES IN LOUISIANA

Introduction

Few studies have examined the prevalence of fecal shedding among active racehorses. An examination of these horses would be of special interest to owners, trainers and veterinarians that care for these animals and express concern about the incidence of diarrhea among this population. Racehorses are subjected to stresses through heavy training, frequent racing, and prolonged transport. In addition, antimicrobials are frequently administered to these individuals, leading to potential alterations in intestinal microflora. Finally, information on fecal shedding among racehorses would be valuable knowledge for the veterinary teaching hospitals that serve as referral centers for these patients.

The primary objective of this study was to determine the prevalence of *Salmonella* in horses at the four racetracks in Louisiana by means of serial fecal culture samples. Additionally, the benefit of culture enrichment techniques on improving the likelihood of detecting low levels of *Salmonella* was examined. Polymerase chain reaction based assay testing was utilized to verify the negative culture results and improve the sensitivity of our testing by detecting false negative culture samples. Finally, the effect of Salmonella status of horses from the different racetracks was evaluated to determine if differences between these sites existed.

The primary biological hypothesis evaluated in this study was that the prevalence of *Salmonella* in racehorses from Louisiana racetracks would be ≥5%. This expected prevalence is greater than that published in the USDA/APHIS/VS survey of the general horse population [67]. The basis for this difference was based on the following criteria: (1) multiple stress factors imposed on this target population compared to the general population which could increase the prevalence of *Salmonella* infection, (2) compared to other horse populations (e.g. pleasure

horses) this population of animals are maintained at high densities under close contact. (3) the management of horses at a racetrack is different compared to pastured animals since they are maintained in confinement and have more direct contact with fecal wastes (4) by acquiring serial fecal samples this study would increase the likelihood of detecting asymptomatic carriers.

Materials and Methods

Selection of Horses – Horses from the four racetracks in Louisiana were sampled during the 2003 or 2004 racing seasons. The racetracks evaluated in this study included Fairgrounds Racetrack in New Orleans, Louisiana Downs Racetrack in Shreveport, Evangeline Downs Racetrack in Lafayette, and Delta Downs Racetrack in Vinton.

The required number of horses sampled from each racetrack (n) was estimated at a 99% confidence assuming a prevalence of ≥ 5%. The formula used to estimate the required sample side was:

$$n = \{1 - (1 - a)^{1/D}] [N - (D-1)/2]$$

whereby "a" was the probability or confidence of observing at least one *Salmonella* positive, "D" was the number of *Salmonella* positive in the population, and "N" was the total population. [111]

Fecal and Data Collection – Trainers who volunteered to participate in the study allowed their horses to be sampled. Two fecal balls were collected from the stall floor using latex gloves and placed into a clean fecal cup. Fecal samples were transported to the laboratory and analyzed within twelve hours of collection. Three serial fecal samples were collected from each horse over a period of five days (Day 1, 3, 5). Originally, it was our intent to collect data regarding age and gender for individual animals; however, we elected not to do so to maintain owner/trainer anonymity and limit potential liability

Fecal Bacterial Culture – Fecal samples were mixed thoroughly and one gram of feces placed in selenite enrichment broth. Cultures were incubated at 37°C for 36 hours under aerobic

conditions. A subculture of selenite broth was inoculated onto xylose-lysine-tergitol 4 (XLT4) agar[a] and incubated under the same conditions, described previously. The presence of bacterial colonies on the culture plates was noted at 24 and 48 hours. Presumptive *Salmonella* colonies were further identified using biochemical reactivity testing with triple sugar iron agar (TSI), lysine iron agar (LIA) and urea agar. A commercial biochemical identification system (API 20E)[b] for Enterobacteriacae was used to confirm the identity of the *Salmonella* isolates according to the manufacturer's instructions. Suspect colonies were also tested for agglutination using polyvalent and O group specific antisera[c]. Growth from TSI agar was inoculated onto stock agar for long-term maintenance of cultures.

Delayed Secondary Enrichment (DSE) – The selenite enrichment cultures were held for five additional days at room temperature to promote further bacterial growth. Five milliliters of broth was inoculated into 7 ml of fresh selenite media and incubated using the same protocol. Bacterial isolates were identified as previously described.

DNA Extraction – Supernatants from the DSE cultures were held at -20°C until testing. Two hundred microliters from each of the three samples for a given horse were pooled. *Salmonella* deoxyribonucleic acid (DNA) was extracted using a commercially available DNA extraction kit using the manufacturer's instructions[d]. The assay was optimized to increase the DNA yield according to the manufacturer's recommendations by increasing the incubation time before elution to 10 minutes and repeating the elution step. DNA quantification was determined by measuring absorbance at 260 nm using a spectrophotometer[e], whereby absorbance of 1 unit at 260 nm corresponded to 50 μg of DNA per milliliter. DNA extracts were held at -20°C for long term storage.

Controls – Five consecutive fecal samples were collected from a healthy horse and were found to be culture-negative for *Salmonella* spp. One gram of feces from the culture negative

horse was inoculate into selenite enrichment media and incubated at 37°C for 36 hours. For positive and negative controls, isolates of *Salmonella* Anatum and *Escherichia coli* organisms, respectively, were grown on sheep blood agar to obtain solitary colonies. Bacteria were diluted into sterile saline and the concentration adjusted to approximate 10^8 CFU/ml based on optical density using spectrophotometer at 625 nm wavelength. One milliliter (10^8 CFU) of bacteria was centrifuged and resuspended in 200 µl of enriched fecal culture and DNA was extracted as previously described. DNA extracts from *Salmonella* Anatum and *Escherichia coli* were serial diluted from 1: 1 x 10^{0} to 1: 1 x 10^{-7} and amplified by PCR.

Clinical Samples – Fecal samples were collected from horses that were admitted to the isolation ward of Veterinary Teaching Hospital at the Louisiana State University during March 2006. A portion of the feces were submitted to the Louisiana Veterinary Medical Diagnostic Laboratory for bacterial culture, using standard methods (described previously). From the remaining fecal specimen, one gram of feces placed in selenite enrichment broth and incubated at 37°C for 36 hours under aerobic conditions. Two hundred microliters of supernatant fluid was held for DNA extraction and PCR amplification (as previously described).

Polymerase Chain Reaction – A highly conserved 457 base pair (bp) nucleotide sequence within the invasion gene (*invA)* of *Salmonella* spp was targeted for amplification.[86] The primer sequences for the oligonucleotides were: the upper strand – 5' TGC CTA CAA GCA TGA AAT GG 3'; the lower strand – 5' AAA CTG GAC CAC GGT GAC AA 3'.[86] A PCR mixture was prepared consisting of 50mM Tris-HCl, (pH 8.3), 25 mM $MgCl_2$, 10µM dNTP (2.5 mM of each dATP, dCTP, dGTP, and dTTP)[f], 0.49 mM of each upper and lower primer[g], 0.1 µg/µl of bovine serum albumin[h], and 5 U/µl *Taq* DNA polymerase[f]. Forty five microliters of PCR reaction mixture was added to 5 µl of sample DNA per reaction. The reaction was performed for forty cycles of 40 seconds at 95°C, 40 seconds at 52°C and 60 seconds at 72°C in a

thermal cycler[i]. After the last cycle, the mixture was incubated at 72°C for 5 minutes. The products of PCR were electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 µg/µl), and were visualized and digitized using ultraviolet imaging[j]. Samples that contained a visible 457 bp fragment were considered test positive (Figure 4).

PCR Product Sequencing – Nucleotide sequencing was performed to assess whether the amplified PCR product was the predicted genomic segment. Purification of PCR product was performed by use of commercial assay according to the manufacturer's instructions[k]. DNA sequencing was performed using a multicolor fluorescence-based DNA analyzer[f]. Resulting sequence was compared to published gene data[l].

Analysis of Results – The 95% binomial confidence interval (CI) of each prevalence estimate was calculated. Where the prevalence estimate was zero, the 95% confidence interval was calculated according to van Belle and Millard.[112] Statistical analysis was performed using EpiInfo 2000[n]. McNemar's test was used to determine if there was a difference between the results obtained using PCR and culture. A kappa test was used to estimate the level of agreement between the two tests. Because 50% of the cells had expected results <5, a 4 x 2 exact test was used to determine if there was a difference in the *Salmonella* status of horses at the four different racetracks. If a difference was found, then Fischer exact tests were used to compare the *Salmonella* status between individual racetracks.

Results

Fecal samples were collected from selected horses at Fairgrounds Racecourse (FG) in New Orleans (January, 2003); Louisiana Downs Racetrack (LD) in Shreveport, (September 2003); Delta Downs Racetrack (DD) in Vinton (January 2004); and Evangeline Downs Racetrack (ED) in Lafayette (June 2004) (Table 2). The sampling period was chosen in the

middle of the racing season, when the population of horses housed at the racetrack was stable. The only breed sampled was Thoroughbred.

The total population of horses at each racetrack prior to the onset of the sampling period was based on estimated stall census records according to the racetrack stewards. At the time of each sampling period there were an estimated 1,800 horses housed at FG racecourse, 1,300 housed at LD, 1,200 horses housed at DD and 1,100 horses housed at ED. Roughly, 40-44 horses were housed in a given barn, and in most cases multiple trainers occupied a single barn. The calculated sample sizes required from each racetrack were similar (Table 2). To ensure the sample size was met, the number of horses initially enrolled was larger (52-72%) than this estimate, and included 138 horses from Fairgrounds, 136 horses from Louisiana Downs, 119 horses from Delta Downs, and 119 horse from Evangeline Downs, or a total of 512 individual horses (Table 2). Individual horses were eliminated from the study if three consecutive fecal samples were not collected. In total, 25 horses were eliminated from FG, 51 horses from LD, 2 horses from DD, and 5 horses from ED; for a total of 83 horses. Horses were eliminated for a number of different reasons, including transfer of ownership (claiming), illness, withdraw of consent by trainer, lack of fecal production, or loss of one or more fecal samples. The final sample size from the four tracks was 113 horses from the FG, 85 horses from LD, 117 horses from DD, and 114 horses from ED. After tabulating the number of horses eliminated from the study, one racetrack, LD, fell below the required sample size (Table 2).

A total of 1,286 fecal samples were collected from the four racetracks and examined by primary bacterial culture and delayed secondary enrichment. A total of 5 horses (1.16%) were positive for *Salmonella* by primary bacterial culture, and included 3 horses from the FG and 2 horses from DD (Table 3). No horses were positive for *Salmonella* spp. from LD or ED by primary bacterial culture (Table 3). After further testing the feces by DSE, 2 horses were found

to be positive for *Salmonella* at LD, but no horses were positive at the remaining 3 racetracks (Table 3). Any given horse was positive for *Salmonella* on only 1 of 3 samples. None of the horses that were positive for *Salmonella* on primary culture were positive on DSE. Additionally, none of the horses that were positive for *Salmonella* on DSE were positive on primary bacterial culture (Table 3). The overall prevalence of *Salmonella* fecal shedding based on all bacterial culture methods was 2.65% at the Fairgrounds, 2.35 % at Louisiana Downs, 1.7% at Delta Downs and 0 % at Evangeline Downs (Table 3).

A total of 429 horses were tested for evidence of *Salmonella* shedding by PCR using the enrichment media from the three samples collected from each horse. Positive DNA amplification for salmonellae, as confirmed by sequencing of PCR product, was found in three horses (0.7%), including 2 from the FG (1.77%) and 1 from LD (1.18%). No horses were positive for *Salmonella* by PCR from DD or ED (Table 4). Only one horse was positive by both bacterial culture and PCR, the remaining two horses that were positive based on PCR were negative for salmonellae by bacterial culture (Table 5). Six horses that were positive by bacterial culture were negative by PCR, even after repeated DNA extractions of individual fecal samples without pooling.

When the data for bacterial culture and PCR was combined, 5 horses were shedding *Salmonella* at FG (4.42%), 2 horses at LD (2.35%), 2 horses at DD (1.7%) (Table 6). The overall prevalence of fecal shedding of *Salmonella* among all the horses tested from the four racetracks was 2.1%. There was a significant difference in the *Salmonella* status between racetracks ($p=0.01$). When examining *Salmonella* status between racetracks, it was found that horses from FG (4.42%) were significantly ($p=0.03$) more likely to test *Salmonella* positive than those from ED (0%). There was no significant difference in the *Salmonella* results between culture and PCR ($p=0.29$). The *kappa* value calculated for the two tests suggest that they have a

high level of agreement (κ=0.97). Primary culture identified 77.5% (5/7) of the *Salmonella*-positive horses. An additional 28.5% (2/7) of the *Salmonella*-positive horses were identified by DSE. Forty-eight percent of the *Salmonella*-positive horses were identified on the first sample, 28.5% by the second sample and the remainder by the third sample (Table 7). The majority of horses were confirmed *Salmonella*-positive on the second and third samples.

Discussion

Results of the present study suggest that the overall prevalence (2.1%, 95% confidence interval [CI], 0.74-3.46%) of *Salmonella* shedding in the racehorse population is lower that the hypothesized prevalence of 5%. However, the prevalence found in this study is slightly higher than the reported prevalence of the general U.S. horse population (0.8 %) [67] and lower than the prevalence among horses surveyed in some veterinary referral hospitals.[7, 57, 60-64, 66, 69, 72-75] By acquiring multiple samples, the current study improved the detection of *Salmonella* shedding among the selected horses by 57%.

Racehorses are a population of horses that are subject to various risk factors that seemingly would increase the acquisition and shedding *Salmonella* organisms. One of the major risk factors for salmonellosis is stress, however defining "stress" is a subject of much debate. Cannon defined stress as the physiologic changes which occur with emotional and physical triggers including increased blood glucose, improved muscle contraction, increased red blood cell numbers, and changes in blood volume distribution. [113] The hallmark of the stress response is activation of the sympathetic nervous system and secretion of catachoamines acutely and cortisol chronically. Another consequence of stress is modulation of the immune cell function and impairment of the immune response to infectious agents. [114] Racehorses are

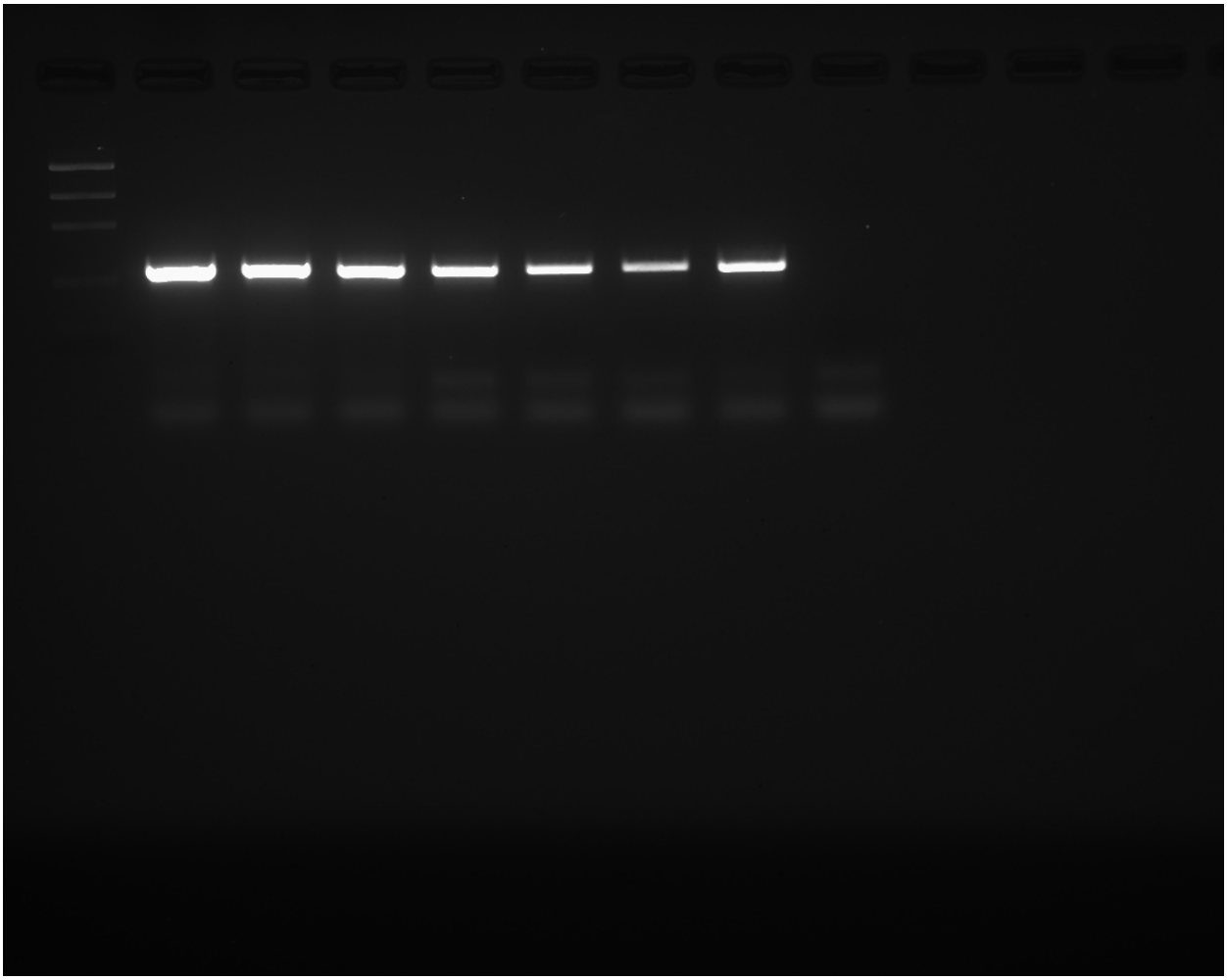

Figure 4: Amplified products in 2% agarose and visualized by UV transillumination after ethidium bromide staining. Various concentrations of *Salmonella* Anatum organism (Lanes 2-7) approximating 10^5, 10^4, 10^3, 10^2, 10^1, 1 CFU respectively were examined to determine the sensitivity of PCR assay for the amplification of *Salmonella invA* gene. Lane 8 contains DNA extracted from a racehorse positive for *Salmonella* by bacterial culture. Amplification of a 457 bp fragment is present from all dilutions of *S.* Anatum and the sample racehorse. Lane 1 is low molecular weight bp ladder and lane 9 contains no DNA template.

Table 2: Results of sample collection of equine feces at four Louisiana racetracks. Horses in which 3 consecutive fecal samples were not obtained were eliminated from the final sample size.

	Collection Date	Estimated resident horse population	Required number of horses	Initial sample size	No. of horses eliminated	Final no. of horses
Fairgrounds, New Orleans	January 2003	1800	79	138	25	113
Louisiana Downs, Shreveport	September 2003	1500	87	136	51	85
Delta Downs, Vinton	February 2004	1200	86	119	2	117
Evangeline Downs, Lafayette	June 2004	1100	86	119	5	114
Total		**5600**	**338**	**512**	**83**	**429**

Table 3: Prevalence of fecal shedding of *Salmonella* spp among horses at four racetracks in Louisiana by bacterial culture. DSE = delayed secondary enrichment.

	No. of horses shedding *Salmonella* by primary culture	No. of horses shedding *Salmonella* by DSE	*Salmonella* prevalence	Estimate of lower 95% confidence interval	Estimate of upper 95% confidence interval
Fairgrounds, New Orleans	3	0	2.65	0.30	5.60
Louisiana Downs, Shreveport	0	2	2.35	0.87	5.57
Delta Downs, Vinton	2	0	1.70	0.64	4.00
Evangeline Downs, Lafayette	0	0	0	0	2.63
Total	**5**	**2**	**1.63**	**0.04**	**2.83**

Table 4: Prevalence of fecal shedding of *Salmonella* spp among horses at four racetracks in Louisiana by PCR

	No. of horses shedding *Salmonella* by PCR	*Salmonella* prevalence	Estimate of lower 95% confidence interval	Estimate of upper 95% confidence interval
Fairgrounds, New Orleans	2	1.77	0	4.20
Louisiana Downs, Shreveport	1	1.18	0	3.47
Delta Downs, Vinton	0	0	0	2.56
Evangeline Downs, Lafayette	0	0	0	2.63
Total	**3**	**0.7**	**0**	**1.49**

Table 5: Comparison of *Salmonella* identification between bacterial culture methods (primary culture and DSE) and PCR.

		Culture		
		Positive	Negative	**Total**
PCR	Positive	1	2	**3**
	Negative	6	420	**426**
	Total	**7**	**422**	**429**

Table 6: Overall prevalence of fecal shedding of *Salmonella* spp by among horses at four racetracks in Louisiana by both bacterial culture and PCR.

	Total number of horses shedding *Salmonella*	*Salmonella* prevalence	Estimate of lower 95% confidence interval	Estimate of upper 95% confidence interval
Fairgrounds, New Orleans	5	4.42	0.06	8.21
Louisiana Downs, Shreveport	2	2.35	0.87	5.57
Delta Downs, Vinton	2	1.70	0.64	4.00
Evangeline Downs, Lafayette	0	0	0	2.63
Total	**9**	**2.10**	**0.74**	**3.46**

Table 7: Number of horses positive for *Salmonella* by bacterial culture (both primary culture and DSE) for the different sampling days.

	Number of *Salmonella*-positive samples		
	1st sample	2nd sample	3rd sample
Fairgrounds	1	1	1
Louisiana Downs	1	0	1
Delta Downs	1	1	0
Evangeline Downs	0	0	0
Total number	**3**	**2**	**2**
	3/7	2/7	2/7
Total percentage	**43%**	**28.5%**	**28.5%**

subject to multiple stresses, both physical and emotional. Physical stressors may include intense muscle exertion from training and racing. Emotional stresses may include frequent transportation, boredom, social dysfunction (both overcrowding and lack of social interaction), equipment or rider fears, and change in environment. [76]

It may be possible that horses that are housed at racetracks may be exposed to various pathogens, including *Salmonella,* through contact with neighboring horses, especially new arrivals and through new environments. Racetracks in the U.S. often house horses for extended periods of time and in high densities during the racing season. In addition, racehorses receive antibiotic administration more frequently that the general horse population due to the frequency of respiratory infections. Change in diet has been associated with increased risk of developing salmonellosis in hospitalized patients. [67] This has included change in the type of hay offered, the addition or deletion of grain or a change in the amount of grain fed. Diets for athletic horses are mainly comprised of energy-rich highly fermentable grains to fuel muscular contraction and performance. Only third to two thirds of the racehorse diet consists of fibrous feeds, mainly hay with little access to grazing or browsing.[115] Additionally, feed is usually restricted for 24 hours prior to racing. Some studies suggest that the composition of the intestinal lumen, which is affected by the ingested foodstuff, affects natural infection with *Salmonella.*[22]

Environmental samples were not acquired in this study, but such information would be useful to determine the potential for *Salmonella* exposure through the facilities. Typically the racetrack housing consisted of 2 rows of abutting stalls that were composed of wood with dirt flooring. Some studies evaluating the recovery of salmonellae from veterinary hospitals suggest that wooden surfaces may reduce access of disinfecting agents to environmental pathogens [75], possibly serving as a reservoir for infection. Racetrack stalls are usually stripped of bedding prior to introduction of new horse, but complete disinfection is not routine.

The recruitment of horses was a pitfall of this study that may have led to potential misclassification bias since participation by the trainers and owners was voluntary. Trainers that elected not to participate may have feared a liability for housing a potentially *Salmonella* positive horse. On the other hand, trainers that chose to participate may have perceived a benefit from complimentary diagnostic services. The sample availability also presented a limitation since some enrolled horses were left the facilities due change of ownership and illness. Acquisition of fecal balls from the stall floor could have produce environmental contamination and ideal sampling would have been directly from the rectum. However, this type of sampling was not possible from this population of horses due the inherent risks of injury to the collector and the horses themselves.

The majority of horses that were shedding *Salmonella* were identified on primary culture (77.6%), however two horses from the same racetrack were identified by DSE only. Interestingly, this racetrack was the furthest geographic distance from Louisiana State University School of Veterinary Medicine, and it is possible that organism viability was affected by transportation time (> 4 hours). The inability to isolate *Salmonella* from other specimens on DSE after positive results on primary culture is not completely unexpected. One study evaluating DSE in poultry found differences in the isolation rate with different serotypes. [79] *Salmonella* Pullorum was less likely to be isolated after DSE, while *Salmonella* Enteritidis was more likely to be isolated. Others have also speculated that if *Salmonella* is present and found in large numbers in culture, the bacteria will reach peak growth during early in the incubation and then decline in numbers, possibly during the delayed enrichment. [80] Therefore, DSE may be most beneficial when the organism is small numbers and should always be combined with primary culture.

In the current study, the kappa suggested that culture and PCR were in high agreement, however, there were several samples in which the results of bacterial culture and PCR were discordant. Several horses were identified as *Salmonella* positive based on PCR alone. In the absence of isolating viable organism, it is possible these samples were false positives and represent non-viable *Salmonella* organism. The PCR protocol used in this assay demonstrated a sensitivity and specificity of 100% when examining samples from hospitalized horses with salmonellosis confirmed by bacterial culture (unpublished data). Some serotypes of *Salmonella* possess atypical characteristics, such as the absence of H_2S production or lactose fermentation, that may lead to misclassification with bacterial culture.

Conversely, PCR was unable to detect *Salmonella* in several samples in which were positive by bacterial culture. It is possible that pooling the culture media from three fecal samples created a dilutional effect that reduced *Salmonella* DNA copy number in the PCR reaction. None of the horses in this study demonstrated clinical signs associated with salmonellosis, and studies have demonstrated that horses with diarrhea may shed up to 10^5 *Salmonella* organisms per gram of feces. [56] Although not measured, it is suspected that non-clinical horses shed *Salmonella* at a much smaller rate.

All the samples were frozen for long term storage and both the DNA extractions and PCR assay were conducted on all the samples at the same time. Therefore, it is also plausible that long term sample storage or sample preparation resulted in DNA degradation. Studies comparing the various methods of DNA extraction have demonstrated that the QIAmp DNA Stool kit yielded the most effective extraction in regard to downstream performance in PCR compared to other methods [103]; however, it is possible residual inhibitor of DNA *Taq* polymerase may remain due to variable concentration of PCR inhibitors in individual fecal samples.

One study in poultry, found PCR to be analytically more sensitive than bacterial culture in the detection of *Salmonella* in feces.[116] In this study, *Salmonella* was detectable up to 1 CFU of organism/g of feces with PCR and 10^2 CFU of organism/g of feces with microbiological culture. However, this same author found contrary results when examining *Salmonella* in feces of horses using the same oligonucleotide primers. In this study, the analytical sensitivity of microbiologic culture with enrichment was similar (10^2 CFU of Salmonella sp/g of feces); but PCR assay only detected sallmonellae to 10^3 CFU of *Salmonella* sp/g of feces. [101] Thus, the analytical sensitivity of PCR can be variable and experimental conditions unknowingly may alter the assay outcome.

Finally, bacterial mutations can result in the deletion of genomic segments and researchers have constructed *invA*-mutant *Salmonella* strains [95], which are still capable of invading the intestinal barrier to cause systemic disease. [29] Therefore loss of some virulence factors, like *invA* gene, might not be a terminal event for invading salmonellae. It is possible the our PCR technique, which targeted the genomic segment encompassing *invA* [86] would miss *Salmonella invA*-mutants.

Product Information

[a] Remel, Lenexa, KS

[b] API 20E, Biomerieux, Durham, NC

[c] Antisera

[d] DNA Mini Stool kit, Qiagen, Valencia, CA

[e] Spectrophotometer

[f] Applied Biosystems, Foster City, CA

[g] Gene Lab, LSU-SVM, Department of Pathobiological Science, Baton Rouge, LA.

[h] New England Biolabs, Ipswich, CA

[i] Gene Amp 9700, Applied Biosystems, Foster City, CA

[j] FluorChem 8800, Alpha Innotech, San Leandro, CA

[k] EdgeBioSystems, Gaithersburg, MD

[l]BLAST, www.ncbi.nlm.nih.gov.

[m]Low mass DNA ladder, Invitrogen, Carlsbad, CA

[n]Centers for Disease Control and Prevention Atlanta, Atlanta, GA

CHAPTER 4

GENERAL CONCLUSIONS

This study provided the first insight into the epidemiology of *Salmonella* shedding in racehorses from Louisiana. Surprisingly, the prevalence of *Salmonella* among racehorses in Louisiana is relatively low, despite several risk factors that would seemingly place this population at higher risk for acquiring and shedding the organism. This information may be useful in determining the need for isolation and surveillance testing within racetracks. In the future, if the *Salmonella* prevalence becomes unacceptably high, an epidemiologic investigation could be initiated to identify the source of infection (feed, birds, humans, etc.) or the stress factors (shipping practices, race schedules, training techniques, age, sex, etc.) associated with disease and subsequently find ways of reducing *Salmonella* shedding in racehorses.

Furthermore, this study has critically evaluated the various methods for testing the prevalence of *Salmonella* fecal shedding among horses, including primary bacterial culture, delayed secondary enrichment and PCR. This investigation has reviewed the advantages and disadvantages of these individual methods and based on our findings conclude a combination of all three methods, when feasible, would provide the greatest accuracy in assessing *Salmonella* shedding among horses.

Finally, although racehorses in Louisiana comprise a major referral population for veterinary hospitals, they do not appear more likely to harbor *Salmonella* in their resident environment than any other population admitted. However, more studies examining the environmental exposure would be needed to further confirm this conclusion.

REFERENCES

1. Cordy, D., *An outbreak of salmonellosis in horses and mules.* J Am Vet Med Assoc, 1946: p. 20-24.

2. Smith, B.P., *Equine salmonellosis: a contemporary view.* Equine Vet J, 1981. **13**(3): p. 147-51.

3. Bryans, J., EH Fallon, BP Shephard, *Equine Salmonellosis.* The Cornell Veterinarian, 1961. **51**: p. 467-477.

4. CDC, *Salmonella Surveillance: Annual Summary, 2004*. 2005, US Department of Health and Human Services, CDC: Atlanta, Georgia.

5. Helmick, C., PM Griffin, DG Addision, RV Tauxe, DD Juraneck, *Infectious diarrhea*, in *Digestive diseases in the Unites States: epidemiology and impact.*, J. Everhart, Editor. 1994, Government Printing Office: Washington, D.C. p. 85-123.

6. Hennessy, T.W., et al., *A national outbreak of Salmonella enteritidis infections from ice cream. The Investigation Team.* N Engl J Med, 1996. **334**(20): p. 1281-6.

7. Kim, L.M., et al., *Factors associated with Salmonella shedding among equine colic patients at a veterinary teaching hospital.* J Am Vet Med Assoc, 2001. **218**(5): p. 740-8.

8. Dargatz, D.A. and J.L. Traub-Dargatz, *Multidrug-resistant Salmonella and nosocomial infections.* Vet Clin North Am Equine Pract, 2004. **20**(3): p. 587-600.

9. Herbert, K. (2004) *New Bolton Salmonella Outbreak*. The Horse.

10. Rice, D., et al., *Household Contamination with Salmonella enterica.* Emerg Infect Dis, 2003. **9**(1): p. 120-2.

11. Reporter, R., *Reptile-associated salmonellosis - selected states, 1998-2002.* Morb Mort Wkly Rep, 200. **52**(49): p. 1206-1209.

12. Cherry, B., et al, *Salmonella Typhimurium outbreak associated with veterinary clinic.* Emerg Infect Dis, 2004. **10**(12): p. 2249-2251.

13. Dolman, C.E. and R.J. Wolfe, *Suppressing the diseases of animals and man : Theobald Smith, microbiologist*. 2003, Cambridge, MA: Boston Medical Library in the Francis A. Countway Library of Medicine;. xii, 691 p.

14. Todar, K., *Textbook of Bacteriology*, in *Structure and Function of Procaryotic Cells*, K. Todar, Editor. 2004, Universtiy of Wisconsin-Madison.

15. Crosa, J.H., et al., *Molecular relationships among the Salmonelleae.* J Bacteriol, 1973. **115**(1): p. 307-15.

16. Rabsch, W., et al., *Salmonella enterica serotype Typhimurium and its host-adapted variants.* Infect Immun, 2002. **70**(5): p. 2249-55.

17. Baumler, A.J.e.a., *Evolution of host adaptation in Salmonella enterica.* Infect Immun, 1998. **66**(10): p. 4579-87.

18. Andrews-Polymenis, H.L., et al., *Host restriction of Salmonella enterica serotype Typhimurium pigeon isolates does not correlate with loss of discrete genes.* J Bacteriol, 2004. **186**(9): p. 2619-28.

19. Liu, G.R., et al., *Genome plasticity and ori-ter rebalancing in Salmonella typhi.* Mol Biol Evol, 2006. **23**(2): p. 365-71.

20. Oliveira, C.J., L.F. Carvalho, and T.B. Garcia, *Experimental airborne transmission of Salmonella Agona and Salmonella Typhimurium in weaned pigs.* Epidemiol Infect, 2006. **134**(1): p. 199-209.

21. Mitchell, B.e.a., *Reducing airborne pathogens, dust and Salmonella transmission in experimental hatching cabinets using an electrostatic space charge system.* Poult Sci, 2002. **8**(1): p. 49-55.

22. Clark, M.A., B.H. Hirst, and M.A. Jepson, *Inoculum composition and Salmonella pathogenicity island 1 regulate M-cell invasion and epithelial destruction by Salmonella typhimurium.* Infect Immun, 1998. **66**(2): p. 724-31.

23. Meyerholz, D.K., et al., *Early epithelial invasion by Salmonella enterica serovar Typhimurium DT104 in the swine ileum.* Vet Pathol, 2002. **39**(6): p. 712-20.

24. van Asten, A.J., J.F. Koninkx, and J.E. van Dijk, *Salmonella entry: M cells versus absorptive enterocytes.* Vet Microbiol, 2005. **108**(1-2): p. 149-52.

25. Isaacson, R.E. and M. Kinsel, *Adhesion of Salmonella typhimurium to porcine intestinal epithelial surfaces: identification and characterization of two phenotypes.* Infect Immun, 1992. **60**(8): p. 3193-200.

26. Gruenheid, S. and B.B. Finlay, *Microbial pathogenesis and cytoskeletal function.* Nature, 2003. **422**(6933): p. 775-81.

27. Lostroh, C.P. and C.A. Lee, *The Salmonella pathogenicity island-1 type III secretion system.* Microbes Infect, 2001. **3**(14-15): p. 1281-91.

28. Blaylock, B., et al., *Microbiology: loading the type III cannon.* Nature, 2005. **437**(7060): p. 821.

29. Vasques-Torres, A., J Jones-Carson, AJ Baumler, S Falkow, R Valdivia, W Brown, M Le, R Berggren, WT Parks, FC Fang, *Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes.* Nature, 1999. **401**: p. 804-808.

30. McCain, C.S. and K.C. Powell, *Asymptomatic salmonellosis in healthy adult horses.* J Vet Diagn Invest, 1990. **2**(3): p. 236-7.

31. Powell, N.G., et al., *Subdivision of Salmonella enteritidis PT 4 by pulsed-field gel electrophoresis: potential for epidemiological surveillance.* FEMS Microbiol Lett, 1994. **119**(1-2): p. 193-8.

32. Fedorka-Cray, P.J., et al., *Alternate routes of invasion may affect pathogenesis of Salmonella typhimurium in swine.* Infect Immun, 1995. **63**(7): p. 2658-64.

33. Collins, F., et al., *Growth of salmonellae in orally infected germfree mice.* Infect Immun, 1978. **21**(1): p. 41-7.

34. Murray, M.J., et al., *Comparative effects of cholera toxin, Salmonella typhimurium culture lysate, and viable Salmonella typhimurium in isolated colon segments in ponies.* Am J Vet Res, 1989. **50**(1): p. 22-8.

35. Murray, M.J., *Salmonella: virulence factors and enteric salmonellosis.* J Am Vet Med Assoc, 1986. **189**(2): p. 145-7.

36. Jones, S.L. and A.T. Blikslager, *Role of the enteric nervous system in the pathophysiology of secretory diarrhea.* J Vet Intern Med, 2002. **16**(3): p. 222-8.

37. Grondahl, M.L., et al., *Secretory pathways in Salmonella Typhimurium-induced fluid accumulation in the porcine small intestine.* J Med Microbiol, 1998. **47**(2): p. 151-7.

38. Murray, M.J., *Enterotoxin activity of a Salmonella typhimurium of equine origin in vivo in rabbits and the effect of Salmonella culture lysates and cholera toxin on equine colonic mucosa in vitro.* Am J Vet Res, 1986. **47**(4): p. 769-73.

39. Matkowskyj, K.A., et al., *Galanin-1 receptor up-regulation mediates the excess colonic fluid production caused by infection with enteric pathogens.* Nat Med, 2000. **6**(9): p. 1048-51.

40. Smith, B.P., et al., *Equine salmonellosis: experimental production of four syndromes.* Am J Vet Res, 1979. **40**(8): p. 1072-7.

41. Smith, B.P., *Atypical salmonellosis in horses: fever and depression without diarrhea.* J Am Vet Med Assoc, 1979. **175**(1): p. 69-71.

42. Parraga, M.E., et al., *A clinical trial of probiotic administration for prevention of Salmonella shedding in the postoperative period in horses with colic.* J Vet Intern Med, 1997. **11**(1): p. 36-41.

43. Weese, J.S., et al., *Screening of the equine intestinal microflora for potential probiotic organisms.* Equine Vet J, 2004. **36**(4): p. 351-5.

44. Palmer, J.E., Benson, C.E. *Salmonella shedding in the equine*. in *Proceedings of the International Symposium on Salmonella, New Orleans, LA*. 1984. Kennett Square (PA): Americal Association of Avian Pathologists.

45. Ikeda, J.S., et al., *Characteristics of Salmonella isolated from animals at a veterinary medical teaching hospital.* Am J Vet Res, 1986. **47**(2): p. 232-5.

46. Donahue, J.M., *Emergence of antibiotic-resistant Salmonella agona in horses in Kentucky.* J Am Vet Med Assoc, 1986. **188**(6): p. 592-4.

47. Castor, M., et al., *Characteristics of Salmonella isolated from an outbreak of equine salmonellosis in a veterinary teaching hospital.* Equine Vet Sci, 1989. **9**(5): p. 236-241.

48. Hartmann, F.A. and S.E. West, *Antimicrobial susceptibility profiles of multidrug-resistant Salmonella anatum isolated from horses.* J Vet Diagn Invest, 1995. **7**(1): p. 159-61.

49. Hartmann, F.A., et al., *Control of an outbreak of salmonellosis caused by drug-resistant Salmonella anatum in horses at a veterinary hospital and measures to prevent future infections.* J Am Vet Med Assoc, 1996. **209**(3): p. 629-31.

50. Tillotson, K., et al., *Outbreak of Salmonella infantis infection in a large animal veterinary teaching hospital.* J Am Vet Med Assoc, 1997. **211**(12): p. 1554-7.

51. Schott, H.C., 2nd, et al., *An outbreak of salmonellosis among horses at a veterinary teaching hospital.* J Am Vet Med Assoc, 2001. **218**(7): p. 1152-9, 1100.

52. Ward, M.P., et al., *Investigation and control of an outbreak of salmonellosis caused by multidrug-resistant Salmonella typhimurium in a population of hospitalized horses.* Vet Microbiol, 2005. **107**(3-4): p. 233-40.

53. Amavisit, P., et al., *Molecular epidemiology of Salmonella Heidelberg in an equine hospital.* Vet Microbiol, 2001. **80**(1): p. 85-98.

54. Astorga, R., et al., *Outbreak of peracute septicaemic salmonellosis in horses associated with concurrent Salmonella Enteritidis and Mucor species infection.* Vet Rec, 2004. **155**(8): p. 240-2.

55. Waterman, A., *A review of the diagnosis and treatment of fluid and electrolyte disorders of the horse.* Equine Vet J, 1977. **9**(1): p. 43-48.

56. Owen, R., et al., *Studies on experimental enteric salmonellosis in ponies.* Can J Comp Med, 1979. **43**(3): p. 247-54.

57. Cohen, N.D., et al., *Comparison of polymerase chain reaction and microbiological culture for detection of salmonellae in equine feces and environmental samples.* Am J Vet Res, 1996. **57**(6): p. 780-6.

58. Ikeda, J.S. and D.C. Hirsh, *Common plasmid encoding resistance to ampicillin, chloramphenicol, gentamicin, and trimethoprim-sulfadiazine in two serotypes of Salmonella isolated during an outbreak of equine salmonellosis.* Am J Vet Res, 1985. **46**(4): p. 769-73.

59. Hird, D.W., et al., *Risk factors for salmonellosis in hospitalized horses.* J Am Vet Med Assoc, 1986. **188**(2): p. 173-7.

60. Mainar-Jaime, R.C., et al., *Influence of fecal shedding of Salmonella organisms on mortality in hospitalized horses.* J Am Vet Med Assoc, 1998. **213**(8): p. 1162-6.

61. House, J.K., et al., *Risk factors for nosocomial Salmonella infection among hospitalized horses.* J Am Vet Med Assoc, 1999. **214**(10): p. 1511-6.

62. Alinovi, C.A., et al., *Risk factors for fecal shedding of Salmonella from horses in a veterinary teaching hospital.* Prev Vet Med, 2003. **60**(4): p. 307-17.

63. Ernst, N.S., et al., *Risk factors associated with fecal Salmonella shedding among hospitalized horses with signs of gastrointestinal tract disease.* J Am Vet Med Assoc, 2004. **225**(2): p. 275-81.

64. Traub-Dargatz, J.L., M.D. Salman, and R.L. Jones, *Epidemiologic study of salmonellae shedding in the feces of horses and potential risk factors for development of the infection in hospitalized horses.* J Am Vet Med Assoc, 1990. **196**(10): p. 1617-22.

65. Owen, R.A., J. Fullerton, and D.A. Barnum, *Effects of transportation, surgery, and antibiotic therapy in ponies infected with Salmonella.* Am J Vet Res, 1983. **44**(1): p. 46-50.

66. Smith, B.P., M. Reina-Guerra, and A.J. Hardy, *Prevalence and epizootiology of equine salmonellosis.* J Am Vet Med Assoc, 1978. **172**(3): p. 353-6.

67. Traub-Dargatz, J.L., et al., *Fecal shedding of Salmonella spp by horses in the United States during 1998 and 1999 and detection of Salmonella spp in grain and concentrate sources on equine operations.* J Am Vet Med Assoc, 2000. **217**(2): p. 226-30.

68. Ravary, B., et al., *Prevalence of Salmonella spp. infections in cattle and horses from the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine, University of Montreal.* Can Vet J, 1998. **39**: p. 566-572.

69. Begg, A.P., et al., *Some aspects of the epidemiology of equine salmonellosis.* Aust Vet J, 1988. **65**(7): p. 221-3.

70. Bucknell, D.G., et al., *Antimicrobial resistance in Salmonella and Escherichia coli isolated from horses.* Aust Vet J, 1997. **75**(5): p. 355-6.

71. Alinovi, C.A., et al., *Detection of Salmonella organisms and assessment of a protocol for removal of contamination in horse stalls at a veterinary teaching hospital.* J Am Vet Med Assoc, 2003. **223**(11): p. 1640-4.

72. Morley, P., et al. *Surveillance for Salmonella shedding in large animal patients*. in *Proceeding of the American College of Veterinary Intermal Medicine*. 2005. Baltimore, MD: American College of Veterinary Internal Medicine.

73. Ward, M.P., et al., *Evaluation of a PCR to detect Salmonella in fecal samples of horses admitted to a veterinary teaching hospital.* J Vet Diagn Invest, 2005. **17**(2): p. 118-23.

74. Palmer, J.E., C.E. Benson, and R.H. Whitlock, *Salmonella shed by horses with colic.* J Am Vet Med Assoc, 1985. **187**(3): p. 256-7.

75. Ewart, S.L., et al., *Identification of sources of Salmonella organisms in a veterinary teaching hospital and evaluation of the effects of disinfectants on detection of Salmonella organisms on surface materials.* J Am Vet Med Assoc, 2001. **218**(7): p. 1145-51.

76. Donnelly, S.G., L.B. Corbeil, *A note on shedding of Salmonella Tuscon by racing horses.* Prev Vet Med, 1983. **1**: p. 383-388.

77. O'Carroll, J.M., et al., *Effects of sample storage and delayed secondary enrichment on detection of Salmonella spp in swine feces.* Am J Vet Res, 1999. **60**(3): p. 359-62.

78. Malorny, B. and J. Hoorfar, *Toward standardization of diagnostic PCR testing of fecal samples: lessons from the detection of salmonellae in pigs.* J Clin Microbiol, 2005. **43**(7): p. 3033-7.

79. Waltman, W.D., et al., *Use of delayed secondary enrichment for the isolation of Salmonella in poultry and poultry environments.* Avian Dis, 1991. **35**(1): p. 88-92.

80. Nietfeld, J.C., et al., *Comparison of conventional and delayed secondary enrichment for isolation of Salmonella spp. from swine samples.* J Vet Diagn Invest, 1998. **10**(3): p. 285-7.

81. Nolan, L.K., et al., *Detection and characterization of Salmonella typhimurium from a dairy herd in North Dakota.* Vet Res Commun, 1995. **19**(1): p. 3-8.

82. Waltman, W.D., A.M. Horne, and C. Pirkle, *Influence of enrichment incubation time on the isolation of Salmonella.* Avian Dis, 1993. **37**(3): p. 884-7.

83. McClelland, M., et al., *Complete genome sequence of Salmonella enterica serovar Typhimurium LT2.* Nature, 2001. **413**(6858): p. 852-6.

84. Biosystems, A. *PCR optimization: reaction conditions and components*. 2005 [cited 2006 January 16]; Available from: www.appliedbiosystems.com/support/techtools/pcropt/.

85. Rahn, K., et al., *Amplification of an invA gene sequence of Salmonella typhimurium by polymerase chain reaction as a specific method of detection of Salmonella.* Mol Cell Probes, 1992. **6**(4): p. 271-9.

86. Stone, G.G., et al., *Detection of Salmonella serovars from clinical samples by enrichment broth cultivation-PCR procedure.* J Clin Microbiol, 1994. **32**(7): p. 1742-9.

87. Cohen, N.D., et al., *Genus-specific detection of salmonellae using the polymerase chain reaction (PCR).* J Vet Diagn Invest, 1993. **5**(3): p. 368-71.

88. Guo, X., et al., *PCR detection of Salmonella enterica serotype Montevideo in and on raw tomatoes using primers derived from hilA.* Appl Environ Microbiol, 2000. **66**: p. 5248-5252.

89. Gulig, P.A., et al., *Molecular analysis of spv virulence genes of the Salmonella virulence plasmids.* Mol Micriobiol, 1993. **7**(6): p. 825-30.

90. Sharma, V.K., Carlson, S.A., *Simultaneous detection of Salmonella strains and Escherichia coli O157:H7 with fluorogenic PCR and single-enrichment-broth culture.* Appl Environ Microbiol, 2000. **66**(1): p. 5472-6.

91. Makino, S., et al., *Establishment of the PCR system specific to Salmonella spp. and its application for the inspection of food and fecal samples.* J Vet Med Sci, 1999. **61**(11): p. 1245-7.

92. Amavisit, P., et al., *Rapid PCR detection of Salmonella in horse faecal samples.* Vet Microbiol, 2001. **79**(1): p. 63-74.

93. Kurowski, P.B., et al., *Detection of Salmonella spp in fecal specimens by use of real-time polymerase chain reaction assay.* Am J Vet Res, 2002. **63**(9): p. 1265-8.

94. Desai, A.R., et al., *An allele-specific PCR assay for the rapid and serotype-specific detection of Salmonella pullorum.* Avian Dis, 2005. **49**(4): p. 558-61.

95. Galan, J.E. and R. Curtiss, 3rd, *Distribution of the invA, -B, -C, and -D genes of Salmonella typhimurium among other Salmonella serovars: invA mutants of Salmonella typhi are deficient for entry into mammalian cells.* Infect Immun, 1991. **59**(9): p. 2901-8.

96. Arnold, T., et al., *Impact of invA-PCR and culture detection methods on occurrence and survival of salmonella in the flesh, internal organs and lymphoid tissues of experimentally infected pigs.* J Vet Med B Infect Dis Vet Public Health, 2004. **51**(10): p. 459-63.

97. Wilson, I.G., *Inhibition and facilitation of nucleic acid amplification.* Appl Environ Microbiol, 1997. **63**(10): p. 3741-51.

98. Chiu, C.H. and J.T. Ou, *Rapid identification of Salmonella serovars in feces by specific detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay.* J Clin Microbiol, 1996. **34**(10): p. 2619-22.

99. Monteiro, L., et al., *Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori.* J Clin Microbiol, 1997. **35**(4): p. 995-8.

100. Fluit, A.C., M.N. Widjojoatmodjo, and J. Verhoef, *Detection of Salmonella species in fecal samples by immunomagnetic separation and PCR.* J Clin Microbiol, 1995. **33**(4): p. 1046-7.

101. Cohen, N.D., et al., *Genus-specific detection of salmonellae in equine feces by use of the polymerase chain reaction.* Am J Vet Res, 1994. **55**(8): p. 1049-54.

102. Cohen, N.D., et al., *Detection of Salmonella enteritidis in equine feces using the polymerase chain reaction and genus-specific oligonucleotide primers.* J Vet Diagn Invest, 1995. **7**(2): p. 219-22.

103. McOrist, A.L., M. Jackson, and A.R. Bird, *A comparison of five methods for extraction of bacterial DNA from human faecal samples.* J Microbiol Methods, 2002. **50**(2): p. 131-9.

104. Abu Al-Soud, W. and P. Radstrom, *Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat.* J Clin Microbiol, 2000. **38**(12): p. 4463-70.

105. Chen, W., G. Martinez, and A. Mulchandani, *Molecular beacons: a real-time polymerase chain reaction assay for detecting Salmonella.* Anal Biochem, 2000. **280**(1): p. 166-72.

106. Heid, C.A., et al., *Real time quantitative PCR.* Genome Res, 1996. **6**(10): p. 986-94.

107. Farrell, J.J., et al., *Broad-range (pan) Salmonella and Salmonella serotype typhi-specific real-time PCR assays: potential tools for the clinical microbiologist.* Am J Clin Pathol, 2005. **123**(3): p. 339-45.

108. Nam, H.M., et al., *Application of SYBR green real-time PCR assay for specific detection of Salmonella spp. in dairy farm environmental samples.* Int J Food Microbiol, 2005. **102**(2): p. 161-71.

109. Hyatt, D.R. and J.S. Weese, *Salmonella culture: sampling procedures and laboratory techniques.* Vet Clin North Am Equine Pract, 2004. **20**(3): p. 577-85.

110. Rudi, K., et al., *Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples.* Appl Environ Microbiol, 2005. **71**(2): p. 1018-24.

111. Sahai, H., A. Khurshid, *Statistics in Epidemiology: Methods, Techniques, and Applications.* . 1996, New York: CRC Press, Inc. 55-77.

112. van Belle, G.M., Steven P, *STRUTS: Statistical rules of thumb*. 1998, University of Washington.

113. Cannon, W.B., *Bodily changes in pain, hunger, fear and rage*. 2nd ed. 1929, Boston: C.T. Brandford Company.

114. Folsom, R.W.e.a., *Exercise alters the immune response to equine influenza virus and increases susceptibility to infection.* Equine Vet J, 2001. **33**: p. 664-669.

115. Kronfeld, D., Harris, P., *Feeding the equine athlete for competition*, in *The Veterinarian's Practical Reference to Equine Nutrition*, K.N. Thompson, Editor. 1997, Purina Mills, Inc and the American Association of Equine Practioners: St. Louis. p. 61-77.

116. Cohen, N.D., et al., *Detection of Salmonella enteritidis in feces from poultry using booster polymerase chain reaction and oligonucleotide primers specific for all members of the genus Salmonella.* Poult Sci, 1994. **73**(2): p. 354-7.

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